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# PLANT PHYSIOLOGY

## EDITORS

CHARLES A. SHULL

GEORGE W. SCARTH

MAX A. MCCALL

CHARLES B. LIPMAN

WILLIAM H. CHANDLER

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HENRY R. KRAYBILL

BURTON E. LIVINGSTON

EDWIN C. MILLER

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## ERRATA

### VOLUME 13

- Page 121, citation no. 1, for "Asrbury" read Astbury.
- Page 126, figure 2, arrange wave length designations as in figure 1, page 125.
- Page 127, figure 3, arrange wave length designations as in figure 1, page 125.
- Page 128, figure 4, arrange wave length designations as in figure 1, page 125.
- Page 243, and odd numbered pages to page 263, running head, for "INDORMANT" read  
IN DORMANT.
- Page 384, line 2, delete "however."
- Page 384, last line, insert "otherwise" after "have."
- Page 386, line 4 from bottom, insert "direct" before "correlation."
- Page 386, line 3 from bottom, omit dashes and read "cell walls and structural rigidity."
- Page 388, delete citation no. 15.
- Page 390, delete citation no. 35.

MOLISCH, the developer of plant microchemistry, and F. CZAPEK, known for his *Biochemie der Pflanzen*.

In the Czech university of Praha, known as Charles University, not till in the year 1898 was a laboratory for plant physiology founded, and opened in 1900 under the direction of Dr. BOHUMIL NĚMEC. His laboratory was at the beginning very small and very poor; but soon it became very well known, not only in his country but also in foreign countries. Many biologists from all countries are drawn to his laboratory by the attractive personality of Professor NĚMEC. He brought plant physiology into close contact with cytology. NĚMEC obtained his first training in scientific work in the zoological laboratory of Professor Dr. F. VEJDOVSKÝ; and his first publications are in zoology. The knowledge of zoology often proved invaluable in the work of Prof. NĚMEC both in general conceptions, as for instance in the application of zoological microtechnic in botany; and in details, as, *e.g.*, in the study of zooecidia in plants and their relation to physiological anatomy. As early as 1899, it was shown clearly by NĚMEC that there is a wave in the viscosity of protoplasm during mitosis, a fact which, nearly forgotten, was not until thirty years later rediscovered, and then led to a detailed analysis of the cell from the viewpoint of colloid chemistry. NĚMEC was the first investigator to publish measurements of the viscosity of protoplasm (1901) in relation to temperature (measured by the speed of fall of starch grains); and his especially keen biological intuition is shown in the fact that he used intact plants or tissues, and not only sections, in his experiments. These facts lead to the conclusion that the basis of colloid chemistry of living matter and of experimental cytology was laid down in Czechoslovakia about the year 1900 by NĚMEC. He also devoted much time and effort to the problem of the structure of the cell, nucleus, and chromosomes (the first photomicrographs of living chromosomes in ultraviolet radiation were made in collaboration with Prof. KRUIS). The theory of statolith starch grains in geotropic responses, first defined by NĚMEC, was cited and accepted by HABERLANDT, and now as generally known and acknowledged, must be considered in any thorough discussion of geotropism.

Extensive study concerning regeneration is a theme to which NĚMEC at different periods of his work has returned. Not only the regeneration of roots and leaves, but special cases of regeneration in fungi must be mentioned here. In the hard tissue of wood fungi (Polyporaceae) which never before were used in physiological experiments, very good objects of astonishing regenerative capacity and reactivity were found by NĚMEC. The regeneration studies are closely related to experimental morphology in which NĚMEC published some papers concerning development, dorsiventrality, and other problems which are handled not only descriptively but are always discussed and worked through in close relation to physiology.

NĚMEC made many studies on irritability, conductivity, reactivity, and tropisms of plants. In extensive studies on mixoploidy NĚMEC is studying the problem of reactivity of the organism and the principal problems of individuality. The last epoch of work of NĚMEC includes extensive studies about minor elements in plants, based on exact analysis of ash; the experiments are conducted with many collaborators.

The Czech students are obliged to Professor NĚMEC for excellent text books in plant anatomy, physiology, and general biology. Notwithstanding the fact that they are written in the Czech language, their illustrations are praised and highly esteemed in foreign countries.

The bibliography of the works by Prof. NĚMEC is too long to be reprinted here; it is *in extenso* published in *Preslia* 2: 5-12. 1923, and supplemented in *Preslia* 11: 3-10. 1932; 12: 69-70, 117-118, 164-166, 207. 1934; 13-15: 337-338. 1936.

Professor Dr. B. NĚMEC, the first professor of anatomy and physiology of plants at Charles University, can be said to have brought plant science in Czechoslovakia to the peak of its development. He is conducting not only his own scientific work, but he is also incorporating his work and that of his many students into the international fabric of botany. His work has exerted a profound influence upon the development of botanical science during the last forty years, and will continue undoubtedly for many decades as one of the major forces in determining trends of botanical research. All research and experimentation developed rapidly in Czechoslovakia after the World War. The foundation of new universities, schools, and research institutions made possible a great extension of the work. Nearly all botanists working and teaching in Czechoslovakia, and some in foreign countries, are students educated in the laboratory of Professor NĚMEC; and the progress of Czechoslovak plant-sciences can be followed in publications not only in the Czech but also in international scientific journals.

CHARLES UNIVERSITY  
PRAHA, ČESKOSLOVENSKO





# RECOVERY PROCESSES OF PONDEROSA PINE REPRODUCTION FOLLOWING INJURY TO YOUNG ANNUAL GROWTH<sup>1</sup>

C. K. COOPERRIDER

(WITH THIRTEEN FIGURES)

## Introduction

In the plateau region of northern Arizona peculiar geologic formations account for the lack of the living natural waters that are typical of most high mountain districts. They also exert a profound influence on the plant growth, and the character of forage through their effect on soil and available moisture. In the forested areas most affected by these formations animals are particularly apt to browse pine reproduction. Tip moths are also very destructive in places, sometimes over large areas. Thus control of injury to young trees, the future forest, is an important problem in the management of these forest lands.

In order to exercise proper control, it is necessary to know at least something of what constitutes serious injury. This depends not only on the degree to which pine seedlings are damaged but also on the power they have to recover. This recovery phase of the problem was investigated in connection with a long-time range-timber reproduction study. Recovery was found to depend principally on the powers ponderosa pine has to replace lost shoots through the development of extraordinary buds.

## Literature

A review of the literature has revealed but little concerning the power of pines to replace those shoots browsed off by animals. BÜSGEN and MÜNCH (1) say, "In general the tendency to epicormic branch formation is less in conifers than in broad-leaved trees. Our Scots pine opens dormant buds only in extremity, after complete defoliation by caterpillars. A few dwarf shoots with ordinary double needles may then be formed from dormant whorl buds, and the dormant eyes between the destroyed needles of the dwarf shoots may become rosette shoots. . . ."

BÜSGEN and MÜNCH continue their discussion with statements concerning conifers, as follows: "The North American *Pinus rigida*, not infrequently cultivated in Germany . . ., develops preventitious shoots very readily even on old stems." (No statement pertaining to the circumstances of development is made.) These authors state further that "the clothing of the stem with green and the replacement of lost shoots,

<sup>1</sup> Publication of the Senior Range Examiner at the Southwestern Forest and Range Experiment Station, maintained at Tucson, Arizona, by the Forest Service, United States Department of Agriculture, in cooperation with the University of Arizona.

besides being brought about by dormant buds produced normally on the young annual shoots (preventitious buds) may be effected through so-called secondary buds . . . in the axils of leaves or bud scales in which no perfected bud would normally have developed. The power of producing such secondary buds is possessed especially by the spruce, and on this to a great extent depends the inexhaustible power of recovery after cutting or grazing which makes it so suitable a hedge plant."

This "inexhaustible" power of spruce trees to recover was observed years ago on Engelmann spruce (*Picea engelmanni*) on too frequently used sheep bed grounds in the high mountains of northern New Mexico.

When substitute buds were observed on ponderosa pine (1925), it was considered most unusual, for there seemed to be a common belief that loss of terminal buds or shoots cut short the development of young pines and that when the primary leader and lateral were destroyed, recovery depended on the presence of an uninjured lower branch. Before this, in writing of sheep injuries to ponderosa pine in Idaho, SPARHAWK (5) recognized recovery through the ascendancy of a side branch and probably the development of buds also when he said, "An uninjured side branch or a new bud takes the place of the leader. . . ." HILL (3) also recognized the common occurrence of recovery where the leading growth was destroyed but does not describe any recovery process. During recent years several authors, including GRAHAM and BAUMHOFER (2), KIENHOLZ (4), and WAKELEY (6), have described growth from substitute buds following tip moth and frost injury to pines.

### Observations

Unmistakable evidences of substitute bud and shoot development on browsed ponderosa pine seedlings were observed during the late summer of 1925 and again in 1926 while working range study plots in the forests of northern Arizona. In 1927 intensive investigations on the relation of grazing to natural regeneration on cut-over timberlands were initiated in the same district. These studies, which were continued over a period of 10 years, offered the opportunity to determine the processes and circumstances of extraordinary shoot development. In addition, observations were extended to the main pine forest areas of Arizona and New Mexico and to some extent to those of southern Colorado.

Cattle and browsing game animals, as well as sheep and tip moths, did the principal amount of injury to seedlings older than 3 years, whereas cutting off top parts of the younger seedlings was done mainly by rodents. Browsing by livestock and game animals, although common and severe in some districts, was lacking or almost so in other districts.

The replacement of lost current growths through the development of substitute buds or shoots proved to be common. It was also found that

there are several different processes whereby ponderosa pine develops substitute buds and shoots, particular development depending on a number of circumstances or factors, such as time when injury occurred in relation to annual shoot growth, factor causing injury, and the parts of a plant removed or injured.

### Processes of recovery

#### SUBSTITUTE GROWTHS

The replacement of lost shoots on older plants takes place on stubs of shoots left and on the stems from which shoots have been removed.

SUBSTITUTE GROWTHS ON YOUNG SHOOTS.—These may open when needles are left on the stubs of injured shoots through development of (a) needle-bundle buds between the needles of the uppermost bundles left, and (b) rarely through whorl buds and the secondary buds in the axils of scales; but stubs without needles die.

SUBSTITUTE GROWTHS ON OLD STEMS.—Such growths may develop from (a) dormant whorl buds, (b) secondary buds in the axils of scales from which no short shoot sprang, and (c) buds of unknown origin herein discussed under *other substitute growths*.

SUBSTITUTE GROWTHS IN JUVENILE SEEDLINGS.—In plants of the cotyledon stage these growths commonly arise from (a) dormant buds that are a part of the growth point above the cotyledons, and in 1- and 2-year-old plants from (b) dormant buds in the axils of primary leaves. None of these different growths are considered adventitious, because they arise from buds formed in due order or from the dormant eye between the needles of short shoots. This excellent statement concerning adventitious buds is taken from BÜSGEN and MÜNCH: "They must not be confused with the dormant buds . . . , which while they may indeed produce shoots out of proper order, were nevertheless originally formed in due order from cells whose active formation has already ceased. This is the case, for example, with *Begonia* leaves which the gardener cuts off and places in moist sand for propagation purposes."

In order to make clear the different processes of substitute shoot development, the normal growth habits of pines will be briefly presented here.

Pines 3 years and older have two distinct forms of branches or shoots, long and short. Long shoots bear the scale leaves and short shoots (also known as modified stems, dwarf shoots, and spurs) the needle bundles or foliage leaves. There is an entirely different behavior in young pines less than 3 years old. On the first growth above the smooth cotyledons and also on the leading growth of the second year are borne spirally arranged single-toothed leaves known as primary leaves, so closely spaced as to

resemble tufts. Branching begins the third year with the development of long shoots.

**SUBSTITUTE GROWTHS ON YOUNG ANNUAL SHOOTS.**—Unstimulated dormant buds (whorl buds and secondary buds) and likewise the eye initial within each needle bundle remain asleep. Either may awaken when the buds or growth point above them are destroyed. The inconspicuous dormant whorl buds lie close below the shoot's tip, hence they seldom escape being taken with the part of the shoot browsed off by animals. Likewise, if tip moth larvae kill a shoot's tip, the dormant whorl buds are destroyed also. Secondary buds (dormant buds in the axils of scales and from which no short shoots grew) develop only when foliage persists to keep the stub alive, and then only rarely. Needle bundles, on the other hand, are usually left on the stubs of browsed shoots and development of eye initials is common.

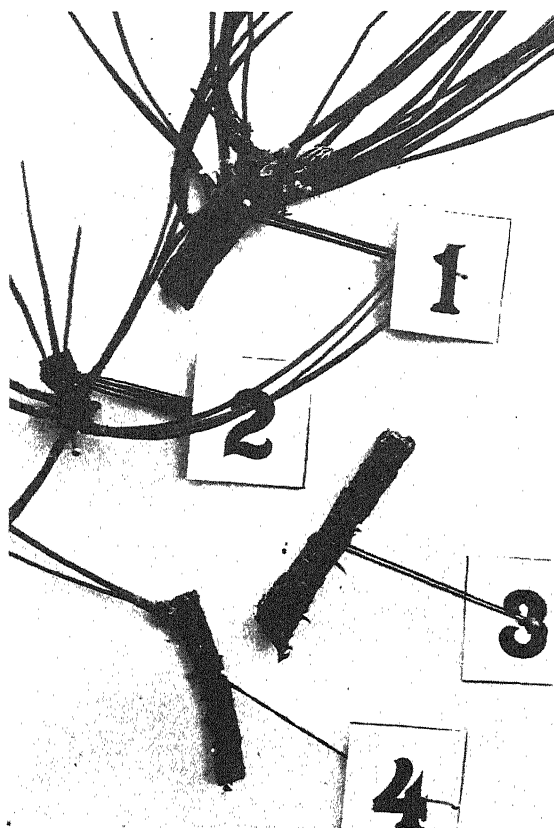


FIG. 1. Stubs of browsed shoots: 1, resin-capped stub with several needle bundles, showing early stages of bud development in the two uppermost bundles; 2, stub with a very young bud (from a small plant); 3, stub (dying for lack of foliage); 4, stub with only one needle bundle in which bud development has begun.

## NEEDLE-BUNDLE BUDS

Browsing animals and tip moths attack young shoots in early summer, mainly during the latter two-thirds of the period of elongation. When browsing, animals jerk off growing terminals, usually leaving stubs 1 or more inches in length with needle bundles. Development of the eye initial in one or more of these soon begins. A similar development takes place following tip moth injury, but the effects of tip moth continue throughout the several weeks' period of development of the larvae in a shoot and subsequent bud development is much less likely to take place. Stubs without needle bundles soon wither and die.

Typical development of needle-bundle buds takes place on stubs of browsed-off young shoots, such as those in figure 1. The stub of a browsed-off shoot exudes a clear sticky resin that soon hardens and covers the injured tissues. The earliest indication of bud development is a slight swelling

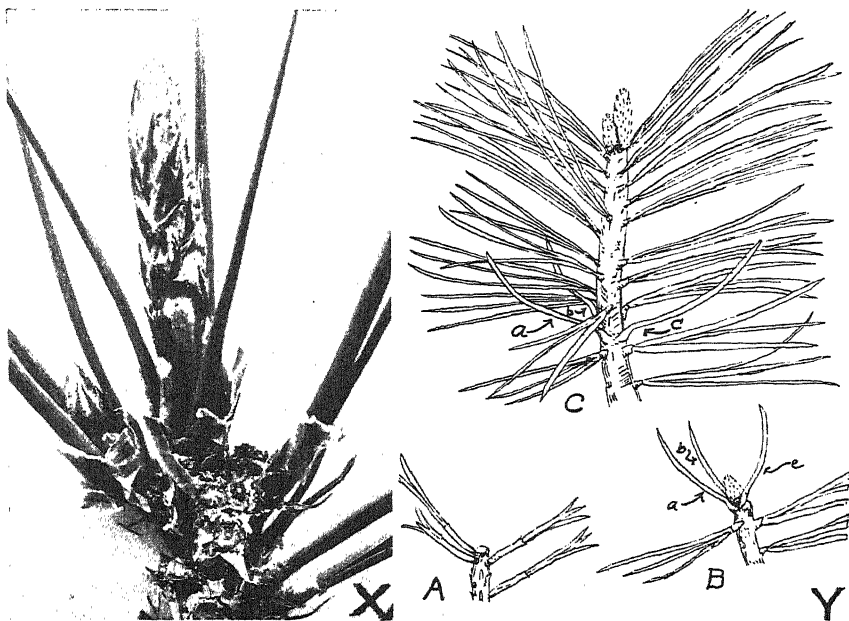


FIG. 2. X. Needle-bundle buds on the stub of a shoot that was browsed in June (photographed in August). The bud on the left is typical of development in the fall, following injury. The bud on the right has begun growth. Some needles were removed to aid photographing. Y. Three stages in the development of an extraordinary bud or shoot from an eye initial. A, stub of a shoot soon after injury, showing the usual stage of needle growth when browsing begins. B, the same stub, in August, with a well-developed needle-bundle bud. C, extraordinary shoot (on stub shown in B) 1 year later. Note the peculiar needles (*a*, *b*, *c*) of the bundle from which the substitute growth opened. Note also how perfectly the new shoot has become a part of the stem.

within the paper-like sheath of one or more of the uppermost needle bundles on a stub. Such development becomes noticeable about 30 or 40 days after injury, but a slightly magnified section of the uppermost needle bundle, the one most likely to develop a bud, will show development of the eye initial in a much shorter time. Buds that appear early in the summer develop rapidly and may elongate into shoots several inches long by October. Current development usually is confined to buds (never more than one to a short shoot), hereinafter called needle-bundle buds which, in the fall, resemble the terminal buds of normal shoots (fig. 2).

During the period of development, such a bud swells, breaks the needle sheath, and pushes the needles apart until they appear to be single leaves on a short shoot which now bears a well-developed bud. Thus such a short shoot has begun to function as a long shoot.

The part of any grazed-off stub above its uppermost needle bundle soon withers and sloughs away. As the young bud develops, it turns upward and, particularly on the stub of a primary shoot, gradually pushes the sloughing tissues above it away and becomes the new terminal bud of the injured shoot (B in Y, fig. 2). Recovery through the growth of such a bud often is so complete that the only indication of past injury is a rind union or crease which resembles a finger-joint line on the palm side of the hand. This crease is formed by the union of the new shoot, which turns upward across the injured end of the stem, with the rind on the opposite side of the old stem from which the needle-bundle bud developed.

Change in function of a short shoot, such as is brought about through development of its eye initial, also is indicated by the needles the shoot bears. These needles attain abnormal size and become so broad at the base as to resemble the leaves of the century plant or agave (X, fig. 2). They set early, whereas normal needles remain soft at the base and may be easily pulled from the sheath so long as they grow, usually until late fall. The needles surrounding a needle-bundle bud also become so fibrous and firmly fixed that they are never shed as are the normal needles but, unless knocked off, persist even after death. Some plants kept under observation retained such needles after 9 years. In fact, these peculiar needles, which become fixed because the short shoot on which they grow becomes a stem and is not shed with the leaves as ordinary short shoots are, furnish unmistakable evidence of former injury. They have been found persisting on the lower part of the main stems of trees, as the one in A of figure 3, that were from 5 to 25 feet tall.

Incidentally, the needles of bundles from which buds spring sometime clearly show that the foliage leaves, which may be short when shoots are browsed, grow from the base and not from the tip end. The needles are particularly apt to adhere to the sheath of a bundle in which bud develop-



FIG. 3. Main stem of a ponderosa pine 5 years after injury from browsing. Note how straight it is. A, single old needle (one of the original bundle shown in B), and, immediately above it, a line where the rind of stub and substitute shoot joined. B, needle-bundle bud through which recovery took place.

ment is taking place. As these needles elongate, they bow outward, being unable to extend in a straight line, burst through the sides of the sheath, and form into loops.

#### DORMANT WHORL BUDS AND SECONDARY BUDS ON YOUNG ANNUAL SHOOTS

Some normal shoots develop well-defined terminal buds early in summer. Injury to such leading buds without destruction of dormant whorl buds seldom occurs. Only a very few shoots have been observed on which the terminal buds had been destroyed by some undetermined factor and substitute buds formed, apparently from dormant whorl buds. In order to test for such a response, the leading terminal and lateral buds were pinched from forward shoots in midsummer. By fall such destroyed buds were replaced by one or more small but otherwise apparently normal buds which opened normal shoots the following year.

A few growths which were considered to be from secondary buds, because of their location in the axils of scales, were observed. They occur only on shoot stubs having foliage, for stubs without foliage die before new growth develops. On stubs with foliage, needle-bundle buds usually develop so readily that secondary buds remain dormant.

Development of dormant whorl and secondary buds on shoots is too rare to be of any practical importance and is described merely to show that such responses do take place.

## GROWTHS ON OLD STEMS

**SUBSTITUTE GROWTHS.**—Old stems as well as current growths may develop substitute shoots. Furthermore, when vigorous plants are pruned severely for several years, the stubs of current shoots with foliage leaves may develop needle-bundle buds and old stems bearing these stubs may develop substitute growth from whorl or secondary buds. Usually shoot development on old stems takes place in extremity, or where no stubs of shoots with needle bundles are left.

When tip moth and browsing animals severely damage young pines, leading terminal and lateral shoots sometimes are killed or pulled off close

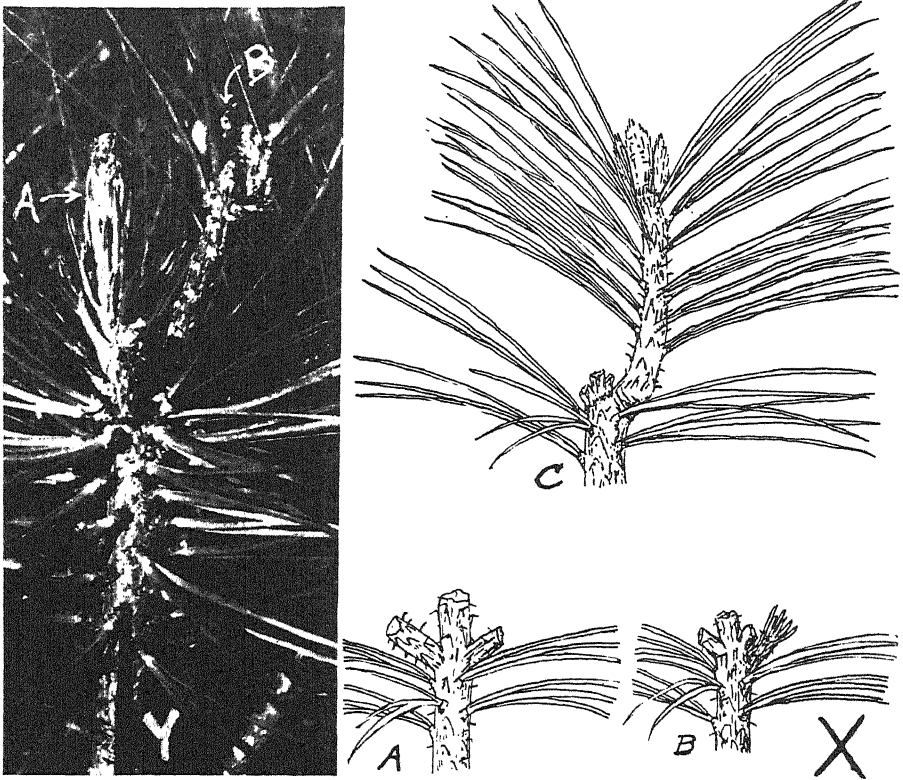


FIG. 4. X. Three stages in the development of substitute growth from a dormant whorl bud. A, main stem with three stubs of browsed shoots without any needle bundles (June). B, new shoot (a) opening from a whorl bud on the old stem (Aug.). The dead stubs have shrunk. C, substitute shoot 1 year later (Aug.). The second year following injury, the main stem was almost straight. Y. Two kinds of substitute growths on a browsed plant. A, new shoot opening from a whorl bud on old stem. B, needle-bundle bud on stub of browsed-off shoot. Note the short growths that resemble primary leaves on shoot A and the needle bundles in the axil of the lower of these leaves. The old stem is that part with mature foliage.



to the old stem bearing them. These lost shoots usually are replaced through development of either dormant whorl or secondary buds on the old stem.

**DORMANT WHORL BUDS.**—Whorl buds open readily from uninjured year-old stems, but where injuries occur year after year stems that have been tipped when they were shoots have no whorl buds. The absence of whorl buds on some stem growths, however, does not prevent recovery, for these buds may develop and open shoots on stems 2 or more years old.

When the growth of the old stem that bears the stubs of lost shoots (A in X, fig. 4) was not injured the previous year, the year it was a terminal shoot, one or more of its sleeping whorl buds located just below the dying stubs is almost certain to awaken. Sometimes both needle-bundle and whorl buds develop, as is the case in Y of figure 4. These whorl bud growths from their beginning resemble elongating shoots. No conspicuous buds form, as in the case of the development of needle-bundle buds. Instead, a cluster of scales and of needle points which otherwise resemble the cone of an elongating normal shoot burst forth, making shoots 1 or more inches long and sometimes much longer the year the plant was injured (B in X and A in Y, fig. 4). These current growths from whorl buds are easily distinguished by their short needles which are crowded on short stem growths, resembling the well-known secondary growth of a normal shoot, which growth also takes place so late in the season that it is stunted by low fall temperature.

Plants that have been injured and have recovered through the growth of dormant whorl buds show that such injuries may be detected with reasonable certainty years after they occur. Whorl bud growths having much longer base than top needles are cone-shaped. Any growth length of the main stem of a plant, which is much shorter than the lengths above and below it and which bears the short crowded needles referred to or their scars, is from a whorled bud, or is normal secondary growth. Such a growth, particularly one without side branches near its base (laterals are uncommon in the case of whorl bud growths), when accompanied by stubs of shoots or their scars and/or a slight crook in the main stem, is unmistakable evidence of shoot injury and recovery through bud development (fig. 5).

Where main stems are the product of uninjured shoots and stubs of shoots, dormant whorl buds may be lacking on stem growths 1 or more years old, because they have been destroyed. In such cases new shoots have been observed on stems 2 or more years old. Wood sections and the location near the tips of annual growths indicated whorl-bud origin of these shoots. Furthermore some records were taken in sufficient detail to determine which stem growths were uninjured when they were current shoots, and thus which ones might have dormant whorl buds.

**SECONDARY BUDS.**—Still other but rather rare-occurring substitute shoots were observed on year-old stems. They occurred on stubs of stems that had

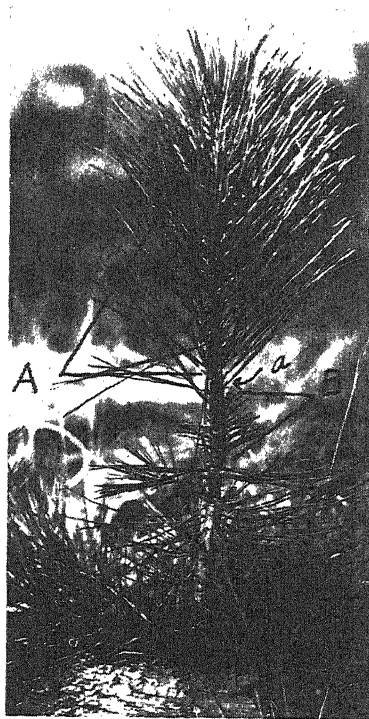


FIG. 5. A many-times injured plant, height 18 inches, illustrating the peculiar appearance of current growth from whorl buds, accelerated growth following release from injury, and recovery. A, shoot growth (1933) from one-half inch stub (*a*) of a browsed (1932) shoot. B, late season whorl-bud growth (1931) following early summer loss of shoots. The visible part of the main stem is the product of two uninjured growths (A and B, both from extraordinary buds) and three short stubs of shoots. The uninjured 1933 growth (A) represents one-half the total height of the 13-year-old tree.

been tipped when they were shoots and from which needle-bundle-bud shoots had opened and been destroyed the following year. Thus such a year-old stem had no whorl buds left and the shoot stub it bore had neither whorl buds nor needle bundles from which substitute growth could develop. These old-stem shoots were considered to arise from dormant secondary buds because of their location with respect to the axils of scales from which no short shoots had developed. To definitely establish the fact, more detailed morphological study than has been made is necessary.

#### OTHER SUBSTITUTE GROWTHS

Small shoots which resemble the slow-growing terminals of oldest side branches appear from time to time on the main stems of young pines below

their oldest side branches. They open on uninjured as well as injured plants, but occur principally before the stem is covered with thick flakes of corky bark. They may last no longer than the foliage leaves they bear or they may put on puny terminal growth for only a year or two. But if the leading growths above are lost, passive main-stem shoots may become active, may put on annual shoots similar to those of normal leading growths, and may become the main stems.

Vigorous shoots similar to those of normal leading growths may also spring from the lower main stems of repeatedly damaged plants. The origin of many of these main-stem shoots seems to bear relation to the location of dormant buds. Such awakened buds push through places where the rind is thin, as scale scars and where the inner bark is exposed in vertical cracks which are so common in the smooth rind of the main stem of young pines. Sometimes vigorous shoots springing from cracks in the rind seem to bear no relation to the probable location of any dormant buds; possibly they are adventitious.

Adventitious budding is also indicated by the opening of shoots from wounds. To illustrate: A small pine was found cut off close to the ground, presumably by a porcupine. The following summer a vigorous shoot appeared above the grass at the mapped location of the cut-off plant. It sprang from the cut surface of the stump's rind but may have grown from a dormant bud. Were this the case, the rarity of their occurrences could be explained by the small number of young pines that are cut off when a stimulus for growth is on and by the poor chance that many of them would be so severed as to expose some dormant bud that still retained its vital connections with the stem.

**SUBSTITUTE GROWTH IN JUVENILE SEEDLINGS.**—Extraordinary budding readily takes place in seedlings in the primary leaf stage. The only animals that appreciably injured seedlings in the cotyledon stage or during the year of germination were rodents, although mortality from other causes, such as drought, was very high.

Rodents, principally ground squirrels and mice, may be very destructive. They cut off the stems of many young plants and all or a part of the cotyledons of others. Most of the injured cotyledons are taken before or while unfolding and in a manner that could not be done by any large-mouthed animal. Small rodents do this when they take the seed coats from germinating plants. This accounts for the fact that any number of the leaflike cotyledons (fig. 6A), from all to only a few, or any amount of all or only a few cotyledons may be cut off, the parts taken depending on the parts still in the seed coat when it is taken.

Obviously when a seedling was cut off below the cotyledons it died, but many rodent-damaged plants with only a few cotyledons or parts of them

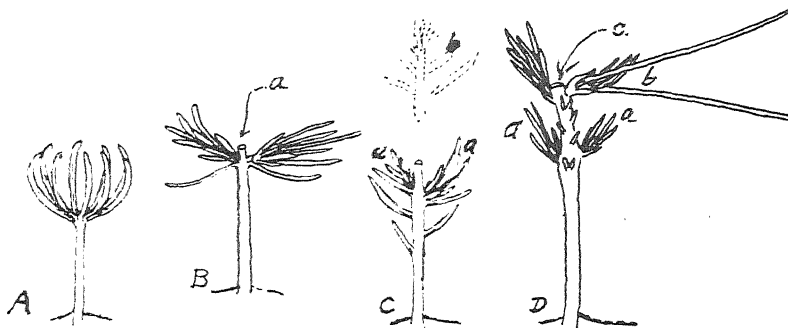


FIG. 6. Growths on young seedlings. A, cotyledon stage (ordinary growth soon after shedding of seed coat). B, substitute growths that are similar to the lost first leading growth. (The first leading growth was bitten off below all leaves (a). The substitute growths, with primary leaves, opened from a point above the cotyledons, which persisted.) C, substitute growths (a) in the axils of primary leaves. (A part of the leading growth (b) of the second year was lost.) D, substitute growths on a three-year-old seedling; a, growths, with primary leaves in the axils of primary leaves; b, a needle-bundle bud. (The plant's first long shoot was bitten off leaving one needle bundle on the stub (c) of that growth and primary leaves on the older growth.)

survived. Where such injuries occur without damage to the plumule, the plant develops normally. The first leading growth above the cotyledons also was commonly damaged by rodents and sometimes by browsing animals. Recovery from such injury took place through the opening of substitute growth at a point above the cotyledons, as is shown in B of figure 6.

#### GROWTH THE SECOND YEAR

Under average conditions, substantial primary growth begins when seedlings are nearly 1 year old. The leading growth above the cotyledons is so nearly covered by primary leaves that even if such growth were cut off by rodents or jerked off by browsing animals, some undamaged leaves usually were left. Buds and then new growths (one bud to a leaf) may open in the axil of one or more of the uppermost of these leaves soon after injury. They open rapidly, forming new growths (C, fig. 6) which also bear primary leaves.

Sometimes when substitute growth is injured soon after it is made, a second growth forms on the remnant of the first and after the same manner as the first. This exceptional ability to produce more than one substitute growth in a season is accounted for by the tendency of pines in the primary-leaf stage to grow throughout the summer rather than to complete growth elongation during early summer, as older plants usually do.

## GROWTH THE THIRD YEAR

During the third year normal seedlings form long shoots from buds nesting in the axil of some primary leaves of the second year's growth. The first long shoots may bear more than one short shoot or may be so small as to have only one with good foliage leaves. As a rule, only shoots of appreciable length are damaged by browsing animals, but tip moths injure smallest shoots also.

When a first long shoot is killed, an undeveloped bud in the axil of some primary leaf opens and a new shoot soon takes the place of the one destroyed. Should the substitute shoot be injured so as to leave a short shoot with foliage leaves, a needle-bundle bud develops after the manner already described, which is shown in D of figure 6.

## SUBSTITUTE BUDDING, TO BE EXPECTED

It seems appropriate at this point to call attention to the relation of extraordinary buds and shoots to the normal life of pines.

As has been pointed out, young pines, beginning with the cotyledon stage, have dormant buds which possess powers of growth similar to those of primary buds.

The needle-bundle bud may be considered the most unusual of the substitute growths, unless possibly pines do produce adventitious buds, and this

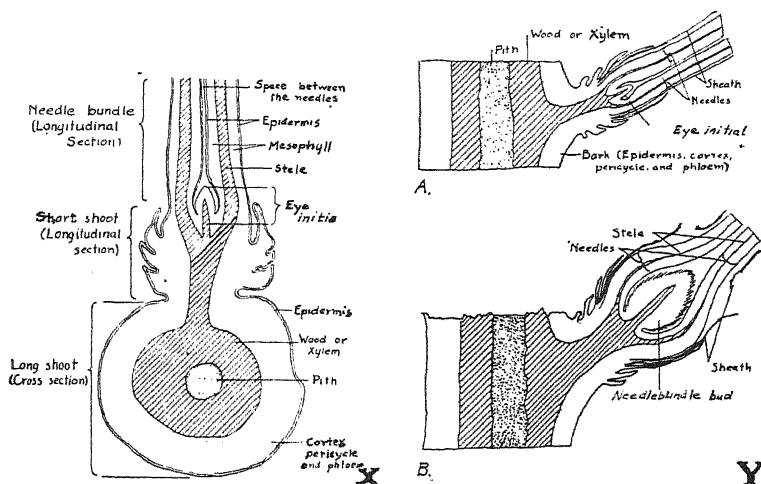


FIG. 7. X, cross-section of a young shoot and longitudinal section of one of its short shoots (ponderosa pine), showing the relation between the eye initial in the needle bundle and the shoot (enlarged 9 diameters). Y, the eye initial a growth point. A, longitudinal section (ponderosa pine) through uninjured long and short shoots, showing the connection between the eye initial and the vital parts of a young shoot. B, the stub of a browsed shoot, showing early stages in the development of the eye initial into a needle-bundle bud. (Enlarged 9 diameters.)

has not been definitely established. But are there any good reasons why development of the needle-bundle bud should be unexpected? A short shoot is but a branch of a long shoot. It retains all its connections with the parent long shoot when current growth and occasionally when 1 or 2 years old. It has an eye initial on its tip. Thus a short shoot has all the parts required for transformation into a long shoot if called on to so function (fig. 7).

At the time these observations were begun, beliefs regarding substitute budding may have been influenced by the behavior of particular pines. Perhaps the tendencies to substitute shoot development may be much less in some species than in others, or some may not form such shoots at all. GRAHAM and BAUMHOFER (2) show that tip-mothed ponderosa pine developed needle-bundle buds less readily than other pines studied at Halsey, Nebraska. On the other hand, men who were shown examples of substitute budding in the Southwest and who suggested that ponderosa pine may have a particular tendency toward epicormic branching, have since found these extraordinary growths on pines in other regions. As a matter of interest, one or more of the forms of substitute shoot development that has been described for ponderosa pine has also been observed in the southwestern yellow pines, Chihuahua (*Pinus leiophylla*) and Arizona (*P. arizonica*).

The suggestion that this tendency may be peculiar to 3-needled pines has also been received. As regards white pines, a few limber pines (*P. flexilis*) occur in the same general region where ponderosa pine was studied. They produce extraordinary growths when injured quite as readily as do pon-

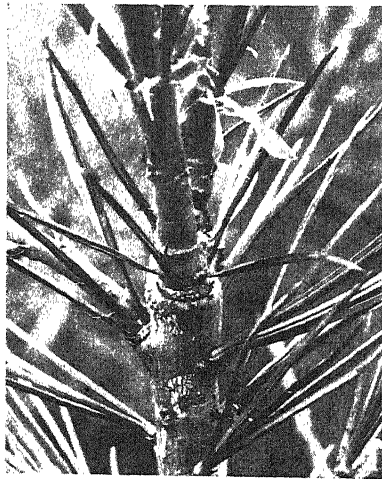


FIG. 8. Extraordinary growths on a young limber pine, which shoots opened from needle-bundle buds, as evidenced by the five needles (characteristic of limber pine) at the base of each shoot.

derosa pine (fig. 8). Such growths have been found on Mexican white pine (*P. strobiformis*) also. So far as is known, there are no other records of this growth phenomenon in white pines.

In ornamental plantings in the Southwest, evidence of recovery from injury through development of needle-bundle buds has been observed on several different unidentified young 3- and 5-needled pines and on the 2-needled Scotch pine (*P. sylvestris*).

#### Factors causing injury, in relation to recovery

Destructive factors, the injuries they do, and the circumstances under which injury takes place will be discussed in other presentations for publication—those presenting the results of the grazing-timber reproduction study previously mentioned. Only summarized statements of findings which show the influence different factors have on extraordinary bud development will be given here.

#### TIP MOTHS

Tip moths deposit eggs on the current growth of pines during the early period of shoot elongation. The larvae, on hatching, eat into and sometimes through the entire length of a shoot. Badly affected shoots die; others die in part (fig. 9). In any event, moth injury influences the chances and processes of recovery somewhat differently than mechanical injury, as from browsing. If only the tip part of a moth-injured shoot dies, as is frequently



FIG. 9. Three tip-mothed ponderosa pine shoots. Left, only the tip was injured, but all except the lower four or five bundles were affected, and they show no evidence of bud development. Right, entirely dead (the three lower needle bundles developed before the larvae reached them). Middle, shoot, nearly full growth, damaged only at tip and side. Such a shoot that attains nearly full growth before its tip dies and that has many unaffected leaves does not, as a rule, develop needle-bundle buds.

the case, needle-bundle buds may or may not open. In contrast to other injuries, the effect of moth injury is progressive and cumulative; it may possibly be toxic. Larvae may be active throughout most of the period of shoot elongation, and any foliage leaves that survive may become stunted and weak. When living but weakened leaves are left on a shoot, no needle-bundle buds usually develop.

The stage of development of pine shoots at the time moth larvae become active may vary considerably from year to year. In some years when moths fly early and their larvae attack shoots during the early period of elongation, the chances of recovery through opening of needle-bundle buds is seriously affected. Such years were 1927 and 1928, when examinations of thousands of moth-injured shoots showed needle-bundle buds on less than 1 per cent. In years when moths fly late, the vigorous and rapid shoot growth of mid-season seems to counteract the damaging effects of larvae and many injured shoots have only destroyed tips or sides, in which event a large percentage of the shoots with undamaged foliage leaves develop needle-bundle buds.

Where shoots are destroyed to the extent that no needle-bundle buds open from them, whorl buds commonly develop on the old stems below the larvae-destroyed leading growth. However, where substitute shoots from whorl buds are moth-injured, the chances of additional substitute growth are greatly reduced, because of the lack of other dormant buds from which such growths might open. The cumulative effect of injuries and the small percentage of recovery through needle-bundle buds indicate how serious tip-moth damage may become. Some tip-mothed plants (in places nearly all of them) on large areas, particularly in warmer parts of southwestern pine forests, remain bushy and deformed until they reach a height beyond which moths ordinarily fly. However, most tip-mothed trees apparently do recover ultimately.

Tip moths are particularly destructive to small plants during the first few years of long-shoot growth; for their shoots are small and any affected shoot usually is completely destroyed. Small plants soon become bushy on account of recurring injury to leading growth and regrowth through opening of numerous substitute buds.

#### BROWSING ANIMALS

Browsing animals took only young shoots and, excepting deer, browse in about the same manner and during the same period.

Any browsing of consequence by cattle, sheep, and antelope occurs during dry periods. It begins in early summer on the most advanced shoots after they have made substantial length growth, but while their needles are still short, as those in A of Y figure 2. Browsing continues throughout the early summer dry period, but ceases with the first summer rains, regardless of the



stage of shoot development. During occasional years of few and irregular summer rains, browsing recurs to some extent during any extended dry period between rains. In most years the period of pine browsing extends from about mid-June to early July.

The earlier shoots are browsed during the annual growth period, the sooner substitute shoots and buds appear and the more pronounced is their development. Where leading growth is browsed off after shoot elongation is complete, either needle-bundle or old-stem buds may fail to develop, at least to any noticeable degree during the same year. The growth of buds that are but slightly stimulated is tardy the following year and the shoots that open from them sometimes are weak and persist as side branches only. Thus, late injury and consequent weak substitute growth tends to produce the greater temporary deformities in the plant.

Deer, in contrast to other animals studied, may destroy terminal buds soon after shoot elongation begins in spring. On parts of experimental ranges where deer congregated and where fresh deer tracks but no sign of other browsing animals could be found, shoots were browsed in May during the early stages of growth before livestock were on these ranges.

Similar findings and general observations over a period of years showed that deer browse pine shoots earlier than other animals. As the season advanced, deer-browsed shoots became difficult to identify because livestock were then taking shoots, and deer scattered from the particular areas where spring damage was found. However, observations indicate that deer continue to take shoots throughout the period when other animals browse. The most conclusive proof of this was found in the Kaibab country north of the Grand Canyon. Here deer have browsed shoots just as severely as cattle and sheep ever do and also continue browsing throughout the period of shoot elongation, or at least until the coming of summer rains.

Where deer peel all the developed terminal buds from a stem, the whole shoot, which is then very short, ordinarily is destroyed. Under such circumstances old-stem whorl buds develop rapidly and soon may form extraordinary growths that closely resemble ordinary shoots (fig. 10). Likewise, if the tip of a very small shoot is so taken as to leave one or more of the then slightly developed short shoots, needle-bundle buds may develop and grow into large shoots the same year.

### Test of injury and recovery

Individual plants under controlled conditions were injured mechanically and compared with uninjured plants in order to test the effect of different kinds, combinations, and degrees of browsing where given injuries were repeated on the same plant over a period of years.

Incidental to the purpose and main findings of this study, it was observed that extraordinary budding took place just as it did on browsed plants, so

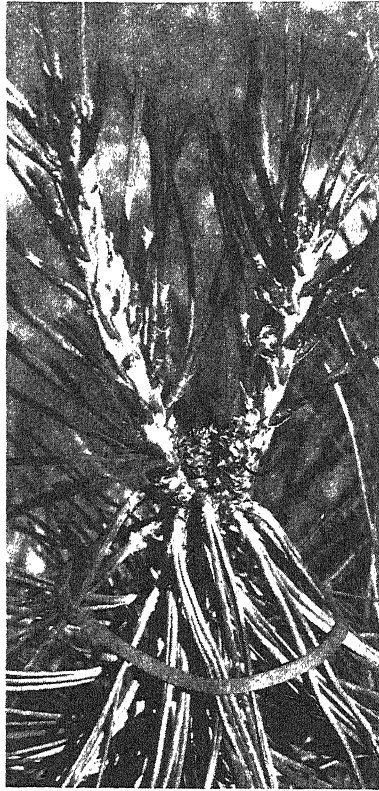


FIG. 10. Young substitute shoots (early August) from whorl buds in which growth was stimulated through loss of terminal buds before shoot elongation began in the spring (late May), showing how such early-growing extraordinary growths resemble ordinary shoots. Deer peeled the swelling buds, leaving the stubs and scales (*a*).

long as shoots were injured at about the same stage of growth and in a similar manner as was done by browsing animals. In short, needle-bundle, whorl, and secondary buds developed when young annual growths were removed mechanically.

Among these mechanically injured plants, the extent of current development of substitute buds or shoots also bore a definite relation to the stage of normal shoot development when injury occurred. To wit, the best buds were produced where shoots were removed before elongation was completed; and among well-developed shoots, only those with immature foliage leaves (needles continue to grow after full shoot length is attained) produced buds the same year. Plants from which leading shoots were removed when both shoots and foliage were fully developed failed to produce noticeable evidences

of buds. On some of these, buds appeared the following year but sometimes failed to open shoots. This shows that substitute buds are awakened most readily and some develop only during the periods when the plant is normally expending stored energy in the production of annual shoot growth. It would seem to indicate that the extent of development of current substitute growth is determined by the amount of unexpended annual growth energy in the plant.

Another interesting and significant observation made on both mechanically injured and browsed plants, and which was demonstrated again and again by the different kinds of growths, is the strong tendency for substitute buds on leading growths to produce leading growths (fig. 11). In most instances shoots from needle-bundle buds gain the lead even where some

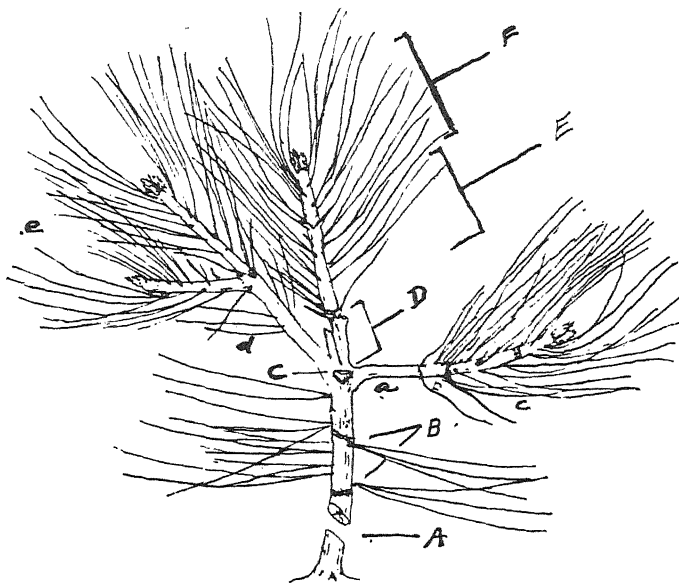


FIG. 11. A many-times browsed ponderosa pine seedling (12 in. high, 10 years old), showing how injured leading shoots tend to produce leading shoots, and how stem length may represent a succession of stubs. A, unsketched part of main stem (3 in.) which carried evidence of several injuries and substitute growths. B, rind creases, evidence of substitute growth following injury. C, dead stub of browsed 1926 growth. D, stub of 1927 shoot from a whorl bud on 1925 growth (stem), following removal of the one leading shoot (C) in 1926. E, stub of 1928 growth from a needle-bundle bud. (Note the peculiar needles at the base of this and other extraordinary needle-bundle growths.) F, 1929 shoot from a needle-bundle bud. The left branch shows uninjured 1927 and injured 1928 growth (*d*). The 1927 shoot was from a whorl bud, as D. The browsed 1928 shoot gave rise to two needle-bundle buds from which opened the 1929 shoots (*e*). Branch to right: *a*, stub of 1927 growth which was produced and injured as D; *b*, stub of 1928 shoot that opened from a needle-bundle bud; *c*, 1929 growth from a needle-bundle bud.

uninjured shoot or branch assumes control for one or more years; and buds on leader stubs are more apt to produce leaders than buds on laterals where both are present. In the absence of needle-bundle buds, uppermost whorl buds (any of which might have been the leading growth in the past) gain the lead when stimulated to make growth. The uppermost bud, either needle-bundle or whorl, on the main stem where substitute buds open on both main stem and branches, is most apt to produce leading growth. The tendency of leading growth to retain the lead is also influenced by the energy the plant has expended in making shoot growth at the time injury occurred. As evidence of this, practically all exceptions to the rule that buds on leading growths produce leading growths were found on plants that were injured too late in the growth period to develop vigorous buds or shoots.

### Substitute growths and recovery of injured pines

This discussion of substitute budding naturally leads to consideration of the bearing these recovery processes have on reforestation. How dormant buds are stimulated and how substitute growths take the place of lost leading growths have been described. What peculiar influence, if any, substitute growths may have on the future life of trees beyond the pole stage can be determined only after a long-time study.

Repeatedly browsed and tip-mothed young pines may be temporarily as bushy as the most common hedge plants. Particularly where juveniles are injured, many substitute shoots are produced for each normal growth that is lost. When such shoots in turn are injured, numerous others open and persist within the margin of the older branches, although any current shoot may be tip-mothed and when it extends beyond the old branches may be browsed. Thus hedged plants build up branches and foliage and gradually gain height. They function and grow through opening of extraordinary buds and shoots which persist through the protection that hedged growth affords (fig. 12). Tests, on the basis of comparison with normal plants of the same age, showed that hedged plants have about as much foliage, both according to number and surface area and total weight of needles as the normal plants, and also a good root system.

The behavior previously described is characteristic of severely injured plants, but most seedlings on any large range, even in districts where browsing is apt to occur, are not injured every year and never become bushes from browsing, although some plants on areas of animal concentration on the same range may be injured for several years in succession. Furthermore, conditions peculiar to some districts where browsing occurs vary so greatly that many pines may be browsed in some years and few in others. Where for any reason plants escape browsing on some years, they open exceptionally large leading shoots the same years and thus maintain reasonably normal growth over a period of years (fig. 13).

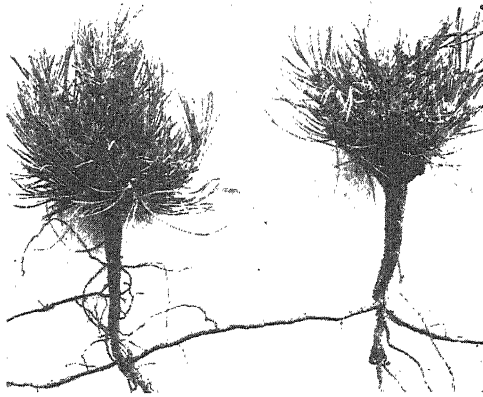


FIG. 12. Two pine bushes about 6 inches high and 9 years old, hedged as the result of many browsing and tip-moth injuries. They show how injured young plants continue growth through the opening of many substitute shoots, and how the stubs of old shoots furnish some protection to the new shoots. Plant on right was severely browsed the year it was photographed; that on left developed a 2-inch leader.

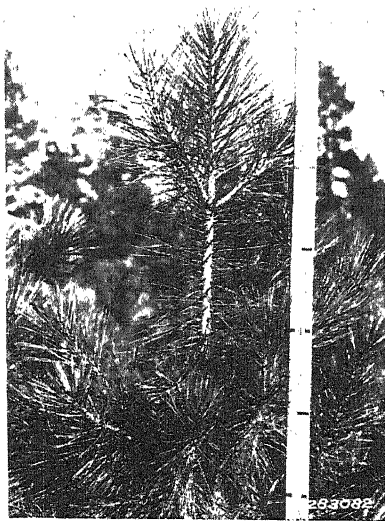


FIG. 13. Accelerated growth made by a formerly short, bushy ponderosa pine. Three-fourths of the total height growth of this plant was made in 3 years, despite the fact that the leading shoot was removed once during that time. On the year following this injury, the long unbranched part of the main stem opened from a needle-bundle bud. The many branches (bush) on the first base foot of the stem died when accelerated growth supplanted the need for the 4- to 7-year-old foliage which had persisted on these branches.

Accelerated growth of hedged plants continues for several years following release from injury, some substitute shoot ordinarily assuming the lead, and, in 3 or 4 years, extending a heavy normally straight stem far above the limits of the hedge from which it sprang (figs. 5, 12, and 13). Almost as soon as such a new main stem has clothed itself with branches and foliage that is in accord with normal plants of its size, the hedged branches at its base cease to function, and die.

The extraordinary ability to produce substitute buds and shoots accounts for the power ponderosa pine has to recover from shoot injury. If it were not for this ability to bud, areas having peculiar conditions that induce animals to browse pines probably could not be grazed by livestock or large game animals at all without jeopardizing future forests. Rodents and tip moths certainly would prevent reforestation in places. The same may be said of the past concerning conditions under which older or mature forest stands developed. When considering the districts in central Arizona below the Mogollon Rim, where nearly all young pine trees up to large pole size are injured year after year by tip moths, one wonders whether many, or even any, of the mature trees escaped injury when they were young. It is fortunate that browsing and tip moth injury affect only current shoot growth and so allow for the development of needle-bundle and dormant whorl buds, and that such injuries occur during periods of normal shoot production when substitute buds are most readily awakened, otherwise injuries would result in much more serious consequences than they do.

But little may be said concerning grazing and timber reproduction relationships in this discussion of recovery processes. The following results are recorded as they indicate the importance of the fact that pines replace lost shoots through a number of different processes of substitute growth.

Of the 2139 ponderosa pine seedlings (about 7 years old in 1927) on 31 plots so scattered as to represent conditions on a 24,000-acre area, 1484, or 69 per cent., were browsed one or more times, some every year during the 9-year period from 1927 to 1935. Of the browsed plants, 2.8 per cent. died. Small rodents injured 171, or 7.9 per cent. of the total number of trees on the same plot areas. Of the 171 injured trees, 127, or 74 per cent., died. Most of the rodent injury consisted of barking or cutting off of main stems.

Of the 5172 recorded seedlings that survived the first 2 months, when mortality from natural causes was tremendous, a total of 74, or 1.4 per cent., were killed by browsing animals and 382, or 7.3 per cent., by rodents. Neither these data nor the statements regarding recovery processes should be interpreted to mean that overgrazing or poor range management may be disregarded. The results of the range-timber reproduction study, which will be presented in future publications, show grave dangers in such practices.

It is well known that trees, particularly hardwoods, when grown from stump and trunk sprouts sometimes are small as compared with seed-produced trees, and may take on bushy or coppice form. No such tendency has been observed in young ponderosa pine poles that developed from extraordinary buds. In this connection, the processes of recovery which have been described take place during the early life of the plant, and substitute shoots actually take the place of lost shoots, rather than form the source of new growth that is superimposed on a part of an old tree, as is often the case with dwarf and coppice growths from sprouts.

SOUTHWESTERN FOREST AND RANGE EXPERIMENT STATION  
TUCSON, ARIZONA

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# UREASE DISTRIBUTION IN *SOJA MAX*<sup>1</sup>

SAM GRANICK<sup>2</sup>

(WITH FIVE FIGURES)

## Introduction

In this account, the distribution of urease, as studied by histological and quantitative methods (10), will be discussed under the sub-headings of the various plant structures.

The activity of the enzyme is expressed in U.U., a urease unit, which is the quantity of urease that will produce 1 mg. of  $\text{NH}_3$  per minute when acting upon urea under the conditions employed in the analyses.

*Soja max* var. Manchu is an upright leafy branching annual legume. The appearance of the plant is much affected by the conditions under which it is grown. In the field, the plants produce a luxuriant growth, forming large leaves, stout, woody stems, numerous pods, and branching is frequent. The plants grown in the greenhouse at Ann Arbor were three feet tall at maturity and rarely branched. The same variety of bean, grown at Woods Hole, Massachusetts, in wooden boxes in soil 6 inches deep, attained a height of only 8 inches and bore 1 to 2 pods per plant. Unless otherwise stated, the determinations described in the following pages were made on plants grown in the greenhouse at Ann Arbor. The reader is referred to the first paper of the series (10) for details as to sampling of material for analysis, etc. The distinctive taxonomic description of *Soja max* var. Manchu is given by ETHERIDGE, HELM, and KING (6).

## Investigation

### COTYLEDONS OF THE GERMINATING SEED

The seed of the soy bean, because of its commercial importance, has been made the subject of numerous chemical investigations. Since data on the chemistry of the seeds were available, it became interesting to attempt to find a correlation between urease and the various chemical substances present in the seed. As was to be expected from the fact that urease is a globulin, the changes of urease were found to be related directly to the general changes in protein content. The cotyledons of jack bean contain much protein and starch. The cotyledons of soy bean are notable for their high protein and fat content, and at maturity contain less than 0.5 per cent.

<sup>1</sup> This is the third of a series of papers on urease distribution in *Canavalia ensiformis* and *Soja max*. For more extensive data and discussion, the reader is referred to the original thesis available at the University of Michigan library.

<sup>2</sup> Newcombe Fellow in plant physiology.

Paper no. 612, Department of Botany, University of Michigan.

starch. Both kinds of seeds possess the highest reported urease activity of any materials yet found. SUMNER (24) estimates that soy beans contain about 0.01 per cent. urease by dry weight.

The histological urease reagents showed that urease was highest in content in the germinating cotyledons. As germination proceeded there was a gradual decrease of the enzyme (figs 1, 2). In the 8-day-old seedlings,

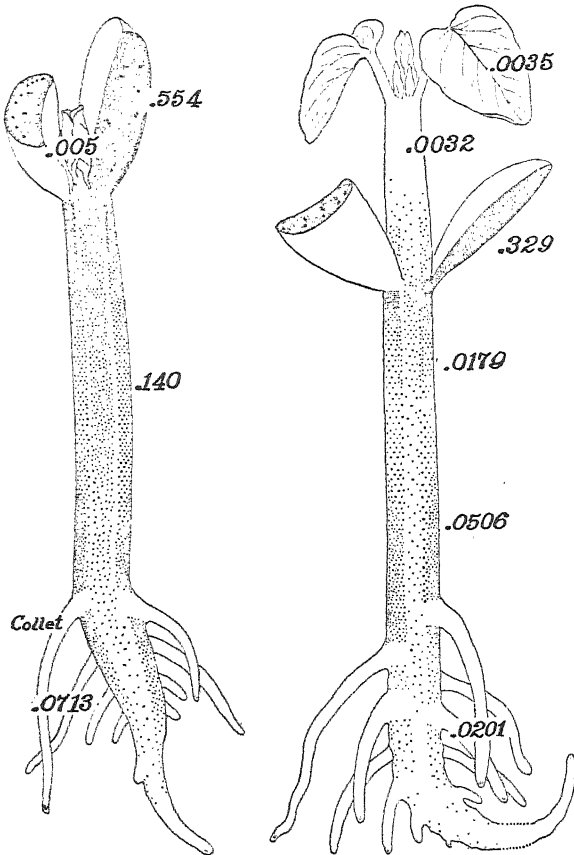


FIG. 1. Composite representation of the urease distribution in the various structures of soy bean seedlings 8 and 15 days old. The number opposite each region of the seedling expresses the number of urease units per gram of fresh weight of that region, as determined by the quantitative method. Stippling indicates the relative urease concentrations as revealed by the histological methods.

the enzyme appeared to be most concentrated at the periphery of the cotyledons, that is, in the subepidermal layers. Urease had diminished markedly in the center of the cotyledons. Some large parenchyma cells gave a stronger reaction for urease than others. The rate of depletion of urease in these

cells showed no evident relation to their proximity to the vascular bundles. The 3 to 4 layers of palisade cells gave a somewhat stronger urease reaction than did the other parenchyma cells. The vascular bundles showed no detectable urease. The content of urease of the bundle sheaths was neither higher nor lower than that of the neighboring parenchyma cells.

Because the chlorophyll content of the outer cells, to some extent, masked the urease reaction, observations were also made on seedlings germinated in

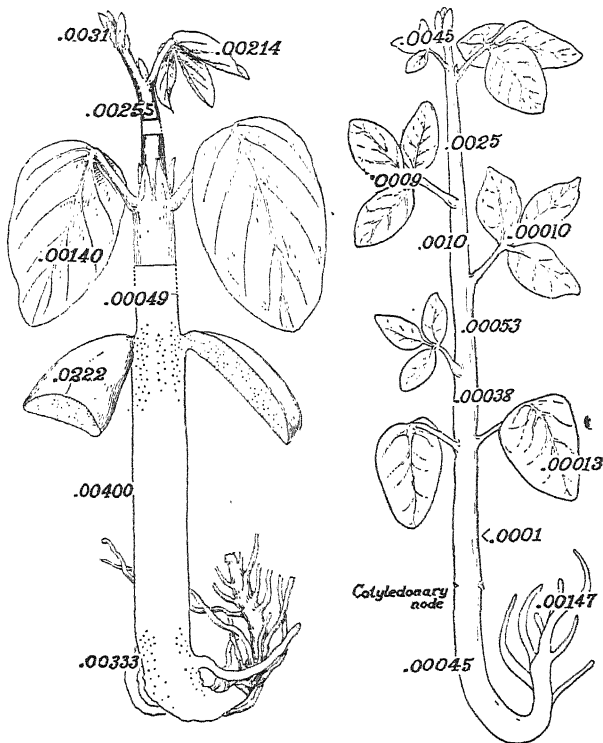


FIG. 2. Composite representation of the urease distribution in the various structures of soy bean seedlings 31 and 52 days old. Numbers have the same meaning as in figure 1.

the dark. There was a more rapid depletion of storage substances from cotyledons of etiolated than non-etiolated seedlings. The urease distribution in the etiolated cotyledons was similar to that in the green cotyledons, except that urease diminished more rapidly in the etiolated cotyledons.

In the 16-day-old seedlings the urease distribution in the cotyledons was similar to that in the 8-day-old plants, except that the total concentration of urease had decreased. The parenchyma cells of the under surface gave a somewhat stronger urease reaction than did the palisade or other parenchyma cells.

In the 30-day-old seedling the cotyledons, which were by now falling off, were yellow, flaccid, and shrivelled. Determinations on these cotyledons generally showed a low urease content.

TABLE I  
UREASE CONTENT OF THE COTYLEDONS OF SOY BEAN SEEDLINGS

TEMPERATURE DURING GERMINATION	DAYS AFTER PLANTING	FRESH WT. IN GM. PER COTYLEDON	DRY WT. IN GM. PER COTYLEDON	U.U. PER GM. FRESH WT.	U.U. PER COTYLEDON
°C.	days	gm.	gm.	U.U./gm.	U.U.
27	0	0.0803	0.0704		
	1	0.1606	0.0693	2.12	0.341
	2	0.1716	0.0688	2.96	0.507
	4	0.1885	0.0554	2.35	0.442
	6	0.306	0.0414	1.48	0.453
20	3	0.176		1.80	0.317
	7	0.215		0.755	0.162
	8	0.222		0.554	0.123
	15	0.356		0.329	0.117
	31	0.263		0.0222	0.00844

A quantitative study was made of the changes in the quantity of urease in the cotyledons at various stages of germination. The changes in urease content taking place at temperatures of 20° and 27° C. during the process of germination and early development of the seedling are shown in table I. These changes are complicated by various factors of which the following are suggested:

1. There will be a decrease of urease as the proteolytic enzymes split the proteins and urease into smaller, more soluble transport products. The urease content will depend then on the activity of the proteolytic enzymes and the rate of transport of their products away from the cotyledons and into the developing tissues.

2. Proteins may be in a storage phase *i.e.*, in aleurone grains, etc. If some urease is present in this state it cannot be detected, for the enzyme must be in a dispersed state in order that urea molecules may come in contact with it (10). Therefore, it is necessary to consider the rate at which dispersal of the stored urease occurs.

3. There is also a possibility that some urease may be synthesized in the cotyledons during germination.

One may consider that during the first days of germination the storage proteins are dispersed and come into solution in the form of soluble proteins at a rate which is more rapid than the rate at which the soluble proteins are

hydrolyzed. The dispersal of proteins is faster at 27° than at 20° C., since water is taken up more rapidly by the soaking seeds at the higher temperature. At 27° C. the urease activity of the cotyledons is greatest after two days germination. The amount of protein being dispersed soon decreases owing to the fact that little storage protein is left; while the dispersed or soluble proteins continue to be hydrolyzed at a rapid rate. Thus a decrease in the amount of soluble protein will take place, appearing as a decrease in urease activity.

#### RADICLE

The *radicle* in the 2-day-old seedlings, was about 1.3 cm. Cross sections were made through various portions of the radicle and their urease content was determined. Sections through the tip of the radicle showed that the *plerome* contained a high percentage of urease; the *periblem* and *dermatogen* gave only a faint reaction. Sections through the middle of the radicle showed that the *epidermis* and *tetrarch xylem* contained no detectable urease; the *pith* was faintly positive; the *cortex* was strongly positive. There seems to be a tendency for urease to disappear more rapidly from the *pith* than from the *cortex*. In the jack bean there appears to be a tendency for urease to decrease more rapidly in the *cortex* than in the *pith*. In general, however, the distribution of urease in the radicle of soy bean is similar to that in the jack bean.

Examination of the *collet* region of 8-day-old seedlings showed a high urease content in the wide *cortex* area and relatively little urease in the *pith* (fig. 1). The *endodermis*, the *cambium*, and the *phloem* and the *xylem* of primary and secondary origin appeared to contain no urease. (The difficulty in judging the slight urease content of a group of cells, adjacent to

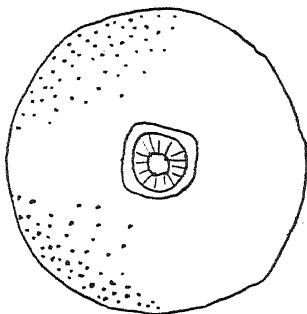


FIG. 3. Urease distribution in a cross section of the collet region of a 39-day-old seedling.

another group of cells containing much urease, has been discussed previously for the collet region of the jack bean.) The bursting of the secondary roots through the cortex causes some of the cortical parenchyma cells at the break to become slightly brownish and to increase in alkalinity to pH 7, or somewhat higher. The young secondary roots were separated from the main root and

tested by themselves. Not more than 50 per cent. of these roots gave positive indications with the urease reagent, and these showed urease to be present at the growing points and nowhere else. Evidently the method is not sensitive enough to detect the low activity of all of these roots. There appeared to be no correlation between the position on the collet axis from which the secondary root arose, and its urease activity at the growing point.

After 16 days urease was still present in the small outer cortical cells. The innermost cortical cells and the stele contained no urease. The collet region loses its urease activity more slowly than any other region. Urease could still be detected in the collet in a 30-day-old seedling but the enzyme was delimited to isolated portions of the outer cortical cells (fig. 3). In the 46-day-old plant no urease could be detected in the collet.

### Root

In the 8-day-old seedling the root is about 7 cm. long. Urease was found to be highest in content in the region just below the collet and to decrease rapidly down the root until within 2 cm. of the tip where it could no longer be detected (fig. 1). The outer cortical cells contained the highest amount of urease at the collet region, but as one descended along the root, the urease reaction became diffuse in the cortex. No urease was present in the stele. At the growing point of the root there was a slight amount of urease which decreased so rapidly that it could not be detected in the elongation region. Generally the primary root tips at this stage all gave the urease reaction, in contrast to the secondary root tips which, on the whole, had a lesser urease content and gave indications of urease in less than 50 per cent. of the secondary roots examined. Very little urease was found in the 15-day-old seedling, in the region of the root just below the collet. Urease therefore decreases quite rapidly with age in the roots, except at the growing points.

TABLE II  
UREASE CONTENT OF THE ROOT

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER STRUCTURE
<i>days</i>		<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
1	Embryo minus cotyledon	0.0122	2.66	0.0324
3	Embryo minus cotyledon	0.0975	0.517	0.0505
4	Root and collet .....	0.105	0.0973	0.0102
8	Root and collet .....	0.131	0.0713	0.0093
15	Root and collet .....	0.274	0.0201	0.0055
31	Root and collet .....	0.419	0.0033	0.0013
52	Root and collet .....	1.36	0.0014	0.0020

In table II are shown the changes in urease content that take place in the root with age. There is a rapid decrease of urease activity per gram of

fresh weight; a rise of total urease per structure to a maximum two days after planting and then a decrease of total urease after that day. Even though the roots have increased rapidly both in weight and number of secondary roots, the total urease content decreases. As will be shown later, this rapid decrease in urease content may be related to the fact that the elongation region of the root is a relatively short one, and the elongating cells mature rapidly.

#### HYPOCOTYL

In the 8-day-old seedling urease had already begun to decrease sufficiently so that differences in concentration could readily be noted in the hypocotyl with the histological urease reagent (fig. 1). The enzyme content was greatest at the cotyledonary node and at the collet. Urease was found only in the parenchyma cells of cortex and pith; never in the cambium or its derivatives. The urease content of the pericyclic cells could not be determined with certainty, although there are indications that these cells do contain urease. Sections through the cotyledonary node showed the pith to contain slightly less urease than the cortex. The enzyme decreased rapidly in the pith from the upper to the lower part of the hypocotyl. At the top of the hypocotyl there appeared to be more urease in the cortical parenchyma cells adjacent to the stele. There was a gradual decrease in urease content in the cortex from the top of the hypocotyl to the lower end, but, on nearing the collet, urease appeared to become more concentrated in the outer smaller cortical cells.

In the 16-day-old seedlings the differences in enzyme content of the various cells had become more marked. The hypocotyl, as seen in cross section just below the cotyledons, still gave strong urease reactions. Owing to elongation there was a mechanical thinning out of the enzyme at this stage, taking place most rapidly in the upper two-thirds of the hypocotyl. In the remainder of the hypocotyl urease became restricted to the outer cortical cells and little of the enzyme was to be found in the pith.

In the 30-day-old seedlings (fig. 2) urease was detected only at the cotyledonary node, and just within the collet region; in both these instances urease was restricted to the outer cortical parenchyma cells. In the 50-day-old plants (fig. 2) no urease could be detected in any portion of the hypocotyl.

Within a period of seven days the hypocotyl had increased from 3 to 60 mm. It will be noted from the data of table III that after the eighth day of germination primary growth and elongation of the hypocotyl had practically ceased. Even before this time the maximum total urease content of the hypocotyl had been attained. The phases of primary growth and maturity overlap. The quantitative data of table III and the results obtained with the histological reagents showed that urease was not only being me-

TABLE III  
UREASE CONTENT OF THE HYPOCOTYL

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
<i>days</i>		<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
1	Embryo minus cotyledon	0.0122	2.66	0.0324
2	Embryo minus cotyledon	0.0975	0.517	0.0505
8	Hypocotyl 6 cm. ....	0.233	0.140	0.0326
15	Hypocotyl 7 cm. ....	0.404	0.0347	0.0140
	Upper half ....	0.194	0.0179	0.00347
	Lower half ....	0.210	0.0506	0.0106
31	Hypocotyl ....	0.434	0.00400	0.00173
52	Hypocotyl ....	0.500	0.00045	0.00020

chanically thinned out, but that already in the late stages of primary growth the enzyme was actually being inactivated or catabolized so that the total urease content of the hypocotyl decreased with further age.

### STEM

The apical meristems possess a urease activity that is too low to be studied with the histological reagent. From quantitative data there is reason for believing that there is no difference in urease distribution between the apical meristems of the soy bean and the jack bean, except the difference in urease concentration.

The epicotyl or second internode<sup>3</sup> is considered apart from the other stem internodes because of its proximity to the cotyledonary node. In the 9-day-old seedling the epicotyl is about 5 mm. long. Cross sections through the tip of the epicotyl, where the first leaves arise, showed urease to be present in the outer cortical parenchyma cells and also faintly in the pith.

In the 16-day-old plant (fig. 1) the epicotyl showed the presence of urease only in the region close to the cotyledonary node; the urease reaction had diminished considerably in intensity. No cells except the parenchyma cells of the cortex, and more faintly the pith, gave a positive test with the urease reagent. In the 30-day-old plant no urease could be detected in the epicotyl.

The quantitative data for the urease content of the epicotyl are given in the table IV. The figures show that the most rapid elongation occurred between the eighth and fifteenth day, during which time the total urease per

<sup>3</sup> For convenience the hypocotyl has been designated as the first internode although this is not technically correct according to the terminology of the morphologist. The epicotyl is considered as the second internode and the other internodes are numbered successively upwards.



structure increased markedly, while the urease activity per gram of fresh weight decreased slightly. Although elongation still occurred after the fifteenth day, it was relatively slight. The phases of primary and secondary growth overlap each other. It may be concluded that the total urease content is at a maximum during the period of the most rapid elongation of the cells of primary growth and thenceforth urease begins to decrease in activity.

TABLE IV  
UREASE CONTENT OF THE EPICOTYL (SECOND INTERNODE)

DAYS AFTER PLANTING	STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER STRUCTURE
<i>days</i>		<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
8	5 mm. plumule .....	0.0116	0.005	0.00005
15	2nd internode 6 cm. ...	0.118	0.0032	0.00030
31	2nd internode 9 cm. ...	0.325	0.00049	0.00015
52	2nd internode 10 cm.	0.509	0.0001	0.00005

Examination of sections made of the other internodes, above the second, and tested with the urease reagents indicated that the amount of urease present even in the youngest internodes was too small to be detected.

Quantitative determinations were made of the urease content of the internodes above the second and the data are shown in table V. Although growth in length is not exactly the same for each internode, (the weight of the sixth internode, for example, being greater than the weight of any older internode) the changes of urease with age are still sufficiently marked to be

TABLE V  
UREASE CONTENT OF INTERNODES OF A 52-DAY-OLD PLANT

STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	TOTAL U.U. PER STRUCTURE
	<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
7th internode including leaflets and apical bud	0.064	0.0031	0.00018
6th internode .....	0.250	0.0025	0.00062
5th internode .....	0.223	0.0010	0.00020
4th internode .....	0.171	0.00053	0.00009
3rd internode .....	0.241	0.00038	0.00008

clearly recognized. These changes in urease content are similar to those undergone by other plant structures. The urease activity steadily decreases with age, the oldest internodes having the lowest urease activity per gram of fresh weight. The total urease per internode is highest in the sixth

internode. This internode has just finished its maximum primary growth. From that time, the total urease content diminishes with the maturation and senescence of the internode.

### LEAVES

In the 1-mm. first foliage or plumule leaves of the ungerminated soaked seed urease was found in all the cells except the primary xylem. The histological reagent also showed that urease decreased rapidly as the leaf expanded.

The data on the urease content of the first pair of foliage leaves of various ages are presented in table VI. The figures in the table indicate the same trend for changes in urease content as was found in the structures previously examined *i.e.*, a decrease of urease activity per gram of fresh weight; and for the total urease content per structure, at first a rise to a maximum which coincides with the period of maximum elongation, then a slow decrease after primary growth has ceased.

TABLE VI  
UREASE CONTENT OF THE FIRST PAIR OF FOLIAGE LEAVES

DAYS AFTER PLANTING	STRUCTURES	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER STRUCTURE
<i>days</i>		<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
8	Plumule—5 mm. ....	0.0116	0.005	0.00005
15	Leaves—3 cm. and petioles—4 mm. ....	0.170	0.0035	0.00060
31	Leaves—4.5 cm. and petioles—1.5 cm. ....	0.387	0.00140	0.00053
52	Leaves—5 cm. ....	0.405	0.00013	0.00005

Sections through the embryonic compound leaves in the apical buds above the second node gave no indication of the presence of urease when the histological method was used. The urease content in the meristematic tissues of the stem, and in the leaf primordia is too low to be detected by this method.

Determinations were made on the trifoliate leaves of soy bean plants grown in the field during the summer, and on others grown in the greenhouse during the winter. The plants grown in the field were harvested at the stage when they possessed pods that were almost mature. The quantitative data (table VII) show the same general trend of urease metabolism as do the plumule leaves. The leaves of the field-grown plants were larger, weighed more, and possessed a urease activity which was maintained at a high level even after primary growth had ceased. The maximum size of the field-grown leaflet was 11 cm. as compared to 6 cm., the maximum size of the

TABLE VII  
UREASE CONTENT OF COMPOUND LEAVES

LEAF	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER LEAF
	<i>gm.</i>	<i>U.U. gm.</i>	<i>U.U.</i>
Greenhouse-grown plants 52 days old			
7th node ..... { 3 cm. leaflets	0.355	0.0045	0.0015
6th node ..... { 6 cm. leaflet	0.777	0.0013	0.0010
5th node ..... { 7 cm. petiole			
5th node ..... { 6 cm. leaflet	0.673	0.0009	0.00060
4th node ..... { 7 cm. petiole			
4th node ..... { 5 cm. leaflet	0.463	0.0001	0.00004
4th node ..... { 7 cm. petiole			
Field-grown plants with nearly mature pods			
16th node ..... { 4 cm. leaflet	0.414	0.00517	0.00214
16th node ..... { 2 cm. petiole			
15th node ..... { 7 cm. leaflet	1.03	0.00467	0.00454
15th node ..... { 5 cm. petiole			
14th node ..... { 9 cm. leaflet	2.28	0.00409	0.00932
14th node ..... { 8 cm. petiole			
13th node ..... { 11 cm. leaflet	3.31	0.00346	0.0114
13th node ..... { 10 cm. petiole			
12th node ..... { 11 cm. leaflet	3.11	0.00264	0.00821
12th node ..... { 10 cm. petiole			
11th node ..... { 11 cm. leaflet	3.98	0.00238	0.00946
11th node ..... { 13 cm. petiole			
10th node ..... { 11 cm. leaflet	3.16	0.00227	0.00718
10th node ..... { 13 cm. petiole			
9th node ..... { 9 cm. leaflet	2.34	0.00294	0.00688
9th node ..... { 8 cm. petiole			
8th node ..... { 8 cm. leaflet	2.09	0.00258	0.00539
8th node ..... { 9 cm. petiole			

greenhouse-grown leaflet, and the weights of each were respectively 3.98 and 0.777 gm. The leaves of the plants grown in the field had a urease activity of 0.005 to 0.002 U.U. per gram of fresh weight, whereas the urease activity of leaves from greenhouse-grown plants was 0.004 to 0.0001 U.U. per gram of fresh weight. If it is assumed that urease activity follows changes in soluble protein content, then it might be said that the greenhouse-grown leaves behaved as if there was a lack of available carbohydrate in the plant for maintaining a high level of protein nitrogen, the depletion of urease and protein taking place rapidly when the leaves had finished expanding because of the drain of carbohydrate and non-protein nitrogen from them into the growing points. However, even under the different environmental conditions of summer and winter growth, expressing themselves in such a wide divergence of growth in green weight and in urease content, the same general scheme of urease metabolism in the leaves was observed as had been noted for the other structures of the plant, namely, that the highest urease

activity was present in the youngest leaves and decreased with age of the leaf; and the total urease content increased during primary growth and then decreased.

#### FLOWER AND FRUIT

The urease content of the flower primordia, the flower, and the pod tissues is below the concentration that is detectable with the histological reagents. The quantitative changes of urease content in the developing pods may be noted from the data in table VIII. The course of the change in urease activity

TABLE VIII

UREASE CONTENT OF POD

STRUCTURE	FRESH WT.	U.U. PER GM. FRESH WT.	U.U. PER UNIT STRUCTURE
	<i>gm.</i>	<i>U.U./gm.</i>	<i>U.U.</i>
Young flowers and buds .....	.....	0.006	.....
Pod (contained 3 mm. seeds) .....	0.1490	0.0051	0.00076
Pod (contained 5 mm. seeds) .....	0.2010	0.0042	0.00084
Pod (contained 9 mm. seeds) .....	0.2518	0.0025	0.00063
Pod (contained 11 mm. seeds) .....	0.371	0.0011	0.0004

is similar to that found for other tissues, that is, there is a steady decrease of urease activity per gram of fresh weight; and an increase and then a decrease in the total content of urease per pod.

#### THE PLANT AS A UNIT

A survey of the changes in the total urease content of the developing seeds and of the seedlings of soy bean is given in a graph (fig. 4). Along the abscissa the relative age of the bean or plant is represented. On the ordinate is plotted logarithmically the total urease values. It may be observed from this graph that the urease content increased almost ten thousand-fold from the youngest seed examined (3.5 mm. long) to the mature seed (13.5 mm. long). Although in the last stages of maturity the seeds were beginning to dry, decreasing in length from 13.5 to 12 mm., and in water content from 63.3 to 48.6 per cent., the urease content still increased. Finally, at the last stage, when the seed had shrunk to 9 mm. and the water content decreased to 19.4 per cent., the urease content had decreased to approximately one-sixth the value of the previous stage.

The completely mature and dry seeds were then germinated. Those planted at 27° C. showed an increase in urease content which reached a maximum at the second day and then began to decrease. Plants grown at 20° C. had a lower level of total urease per plant. There was a rapid decrease of total urease per plant from the fifteenth day.

As with the jack bean, the curves illustrating the changes in total urease content per seed or per plant really indicate the changes of urease taking place in the cotyledons, since these structures contain most of the enzyme. In the developing seed, the cotyledons, especially in the later stages, consti-

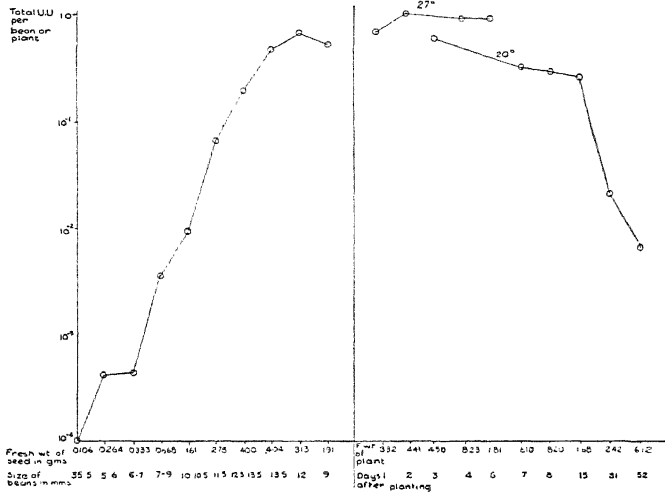


FIG. 4. The graph represents the total urease content of the developing beans, or of the seedlings of soy bean, at different stages of growth. Along the ordinate, total U.U. values are plotted logarithmically; along the abscissa, relative ages of the beans or seedlings are represented.

tute over 90 per cent. of the dry weight of the seeds. As the seeds increase in size, urease increases very rapidly. In the last stages when the seeds begin to dry (as indicated by the pods turning yellow, the hila of the seeds changing from green to pink then to purple and finally to black, and the cotyledons from a green to a yellow-green and finally to yellow) urease increases and finally decreases. This decrease in urease activity is interpreted as a storage of the enzyme in a form which is not readily dispersed in aqueous solution.

In the 31-day-old plant, the cotyledons, which are about to fall off, still contain approximately 80 per cent. of the total urease of the plant. The marked decrease in urease content between the 15-day-old and 31-day-old plant is due primarily to the rapid decrease in urease of the cotyledons themselves. Even after the cotyledons have fallen off, however, the total urease of the plant continues to decrease.

A composite picture of the distribution of urease in the various parts of the developing seedlings of soy bean 8, 15, 31, and 52 days after planting is represented in figures 1 and 2. The stippling indicates the relative urease content as revealed by the histological methods. The numbers opposite each

plant part are the number of urease units per gram of fresh weight of that plant part as determined by the quantitative method.

### Discussion

#### COMPARISON OF UREASE DISTRIBUTION IN SOY BEAN AND JACK BEAN

It was shown in the second paper of this series (11) that, in all the organs and plant structures examined, the urease changes follow the same course. The urease activity of an organ per gram of fresh weight (curve A, fig. 5)

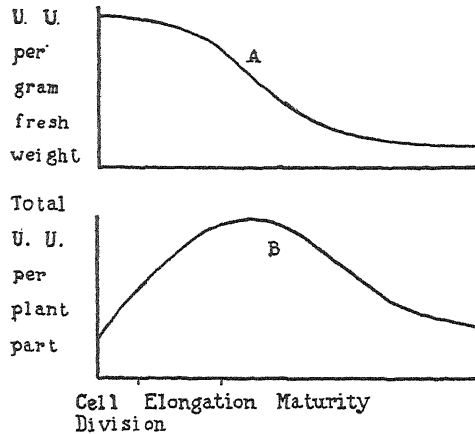


FIG. 5. Changes of urease activity in relation to physiological age. Curve A, change of urease activity, of an organ per gram of fresh weight, with age; curve B, change of total urease content of an organ with age.

is highest when the cells are still meristematic and decreases as the cells grow older. The total urease content of an organ (curve B, fig. 5) increases rapidly during the period of cell division until, at about the time primary growth has ceased, the organ contains a maximum urease content. From that time on, there is a decrease in urease content which is more or less marked, depending on the individual organ considered. It was further shown that the changes in urease activity noted in the organs are due primarily to changes taking place in the parenchyma cells of the organs since neither the cambium nor its derivatives contain detectable amounts of the enzyme. The total urease activity of the meristematic cell increases rapidly at first (curve B, fig. 5); and as the cell passes into the stage of elongation the rate of increase of urease activity drops off. About the time the cell has acquired its maximum size it contains a maximum amount of urease. After this time there is a gradual decline of urease activity per cell until some constant low level is reached. This series of changes found in *Canavalia ensiformis* is likewise found in *Soja max.*

There were, however, some minor differences. In general the urease concentration was less in soy bean than in jack bean. The palisade cells of the soy bean cotyledon contained somewhat more urease than did the other parenchyma cells; and in the 8-day-old seedling, urease was present in somewhat greater concentration at the periphery of the soy bean cotyledons than toward the inner region. In the jack bean cotyledon no such differences in distribution were observed. There was a more rapid decrease in urease activity in the pith than in the cortex of the soy bean hypocotyl during germination. In the jack bean, however, the decrease in the hypocotyl was somewhat more rapid in the cortex than in the pith.

#### UREASE IN OTHER LEGUMES

That the curve of urease changes is not merely limited to the two plants investigated but holds also for other legumes is indicated by the experiments of KIESEL and TROITZKI (12). Their limited data on the urease changes in a number of legume plants are in accord with the general changes of urease in the jack bean and soy bean plants. For example, the young developing seeds of *Phaseolus vulgaris* and *Pisum sativum* contained one-fifth the urease per gram of fresh weight of the older seeds. The leaves of a *Pisum* seedling three weeks old contained more urease per gram of fresh weight than did the stems. Cotyledons of 10-day-old seedlings of *Vicia faba* had about five times the urease activity per gram fresh weight that cotyledons of 28-day-old seedlings had. The changes in urease distribution and concentration found in the soy bean and jack bean may then be considered as changes which are at least common to the Papilionaceae.

#### A COMPARISON OF DISTRIBUTION OF UREASE AND OTHER ENZYMES

BURK (5) has called attention to enzymic activities which are growth-bound, *i.e.*, "where the enzymic activity is correlated with the structure of the living cell to the extent that the velocity of formation of the (intracellular) reaction product parallels and is normally measured by the velocity of growth. Such growth-bound enzymes may be termed phyto-enzymes."

Since the enzyme urease appears to be so intimately bound up with the growth of the parenchyma cell, one may consider urease as a growth-bound enzyme. It was interesting to see whether other hydrolytic enzymes might likewise be growth-bound and undergo changes similar to those found for urease. In table IX data are presented on the analyses of urease, protease, and lipase in the developing seed. The urease content increases markedly during the period of seed ripening. TOVARNITZKY'S analyses (25) of the nitrogenous fractions of soy beans at different stages of development show that the increase of urease activity parallels the increase in the soluble protein content and this investigator calls attention to the fact that "A reliable

# ANALYSES OF SOY BEAN SEEDS AT DIFFERENT STAGES OF DEVELOPMENT

ANALYSES	SIZE OF BEANS IN MM.							
	3.5-5	5-6	6-7	7-9	10-10.5	11.5-12.5	13.5*	13.5†
								13.5†
								12
								YELLOW BEANS DRYING
								9
								YELLOW BEANS DRY
to-N in mg.	2.94	2.84	2.88	2.92	2.34	2.16	.....	5.72
r gm. fresh wt.	12.0	17.1	18.9	13.4	9.87	6.85	.....	7.10
r gm. dry wt.	0.0311	0.0750	0.0960	0.195	0.376	0.600	.....	1.09
NH <sub>3</sub> in mg.								
r gm. fresh wt.	0.156	0.145	0.156	0.124	0.130	0.184	0.135	0.124
r gm. dry wt.	0.588	0.871	1.02	0.568	0.549	0.583	.....	0.154
r bean	0.00165	0.00382	0.00519	0.00282	0.0209	0.0512	0.0545	0.0239
ase activity in mg. NH <sub>3</sub> produced per min.	0.010	0.0164	0.0139	0.0555	0.0596	0.243	1.20	2.92
U. per gm. fresh wt.	0.037	0.0986	0.0891	0.254	0.252	0.770	1.35	3.65
U. per gm. dry wt.	0.00010	0.00043	0.00048	0.00370	0.00958	0.0675	0.485	0.558
ase activity in mg. NH <sub>3</sub> -N produced in 48 hr.	12.85	11.00	11.85	10.60	10.00	7.30	.....	9.40
: gm. fresh wt.	48.4	66.1	77.7	48.6	42.2	23.2	.....	11.7
: gm. dry wt.	0.136	0.290	0.395	0.708	1.61	2.03	.....	1.80
: bean								
ic activity in cc. of 0.1 N acid produced in 48 hr.	3.36	.....	3.40	2.74	3.86	4.68	.....	1.12
: gm. fresh wt.	12.6	.....	22.3	12.6	16.3	14.8	.....	1.39
: gm. dry wt.	0.0356	.....	0.113	0.183	0.621	1.30	.....	0.214
: bean								
ble acidity in cc. of 0.1 N acid	0.432	.....	0.381	0.341	0.352	0.203	.....	0.284
: gm. fresh wt.	0.0106	0.0264	0.0333	0.0668	0.161	0.278	0.313	0.191
wt. per bean	0.00281	0.00439	0.00507	0.0146	0.0382	0.0876	0.404	0.154
wt. per bean	73.5	83.3	84.8	78.1	76.2	68.7	63.3	19.4
ntage water in bean								

Hilum light pink; pod turning yellow.

Hilum dark purple; pods dark yellow; beans yellow green.



index for the amount of protein in soy beans, its solubility and the maturity of the beans, is the activity of urease.<sup>7</sup> As the bean matures, urease accumulates in the cells, some of it being stored in the form of ergastic amorphous substances just as other globulins are stored. The water content in the later stages is decreasing, the cellular materials becoming more and more concentrated; and the semi-fluid vacuoles of the cells take on the appearance of solid spherical corpuscles. Although the storage phase is evident at the last stage (*i.e.*, a decrease of urease activity in the 9 mm. yellow bean) it is highly probable that some storage of the protein urease has occurred even in the earlier stages of seed development.

This increase of urease activity as the seed matures is not paralleled by an increase of protease or lipase activity.<sup>4</sup> It is seen from table IX that the protease and lipase activities per gram of dry weight of seed increase at first, reach a maximum activity in the 6- to 7-mm. bean and then decrease as the bean enlarges. It is interesting to note that the values for amino nitrogen and free ammonia per gram dry weight, and for the percentage of water content are also highest in the 6- to 7-mm. bean. These results on protease and lipase activities of the developing bean do not agree with those of RIVKIND and TOVARNITZKY (25) who report a slight increase of these enzymes in the maturing seed. These investigators made analyses on the air-dried seeds, and do not describe their methods of analyses. The determinations reported in table IX were made on freshly picked material without preliminary drying; corrections were made for the varying values of autolysis, which become important in estimating the very low activities of both protease and lipase found in the soy bean. In this connection it may be mentioned that, in the developing seeds of *Vicia faba*, BLAGOWESTSCHENSKI (3) found that the amylase content per gram dry weight of seed decreased as the seeds ripened.

Upon germination of the seeds the aleurone grains and crystalloids absorb water and are seen gradually to take on a semi-fluid appearance. In the beans grown at 27° C. (table I) the values for urease activity per gram of fresh weight of the cotyledon increased up to a maximum value on the second day and then began to decrease. [The germinated beans, used for the determinations on protease and lipase (table X), were not those related to the developing beans of table IX, so that no direct comparison can be made between the protease and lipase activities of the developing and germinating beans.]

<sup>4</sup> Proteinase activity was measured by permitting aliquots of fresh tissue mash to act on a 2 per cent. casein solution for 48 hours at 30° C. in the presence of phosphate buffer (pH 6.5) and overlaid with toluene. Amino-N was determined by the Van Slyke nitrous acid method. Lipase activity was measured by the increase in titratable acidity of a gum arabic-castor oil emulsion, containing about 2 per cent. fat, when acted upon by 0.5 per cent. tissue mash for 48 hours at 30° C. and overlaid with toluene. (Toluene does not interfere with the determination.) Titration to the phenolphthalein end-point is made on an aliquot of an alcohol-ether extract of this mixture.

The protease activity per gram of fresh weight of the cotyledon reached a maximum in two days and then slowly decreased. From the complete hydrolysis of proteins there will be produced some 7 mg.  $\text{NH}_2\text{-N}$  per bean. This indicates that protein digestion during germination is significantly limited by the low protease activity. Lipase activity increased in two days and was apparently highest in the 11-day-old stage; it then rapidly decreased. In a microchemical examination of the germinating soy bean

TABLE X  
ANALYSES OF COTYLEDONS OF GERMINATING SOY BEANS

	DAYS AFTER PLANTING						
	1	2	5	8	11	15	30
Fresh wt. per cotyledon pair .....	0.274	0.286	0.590	0.512	0.496	.....	0.264
$\text{NH}_2\text{-N}$ in mg. per gm. fresh wt. ....	2.32	2.70	2.98	3.40	1.68	0.861	.....
Protease activity in mg. $\text{NH}_2\text{-N}$ produced in 48 hr. per gm. fresh wt. ....	3.30	4.25	.....	2.70	1.45	0.27	0.078
Lipase activity in cc. of 0.1 N acid produced in 48 hr. per gm. fresh wt. ....	1.48	3.41	2.24	2.39	4.58	0.86	0.00

VON OHLEN (26) reports that in the cotyledons a marked decrease of oil had occurred by the fourth day; depletion began at the base of the cotyledons and progressed toward the opposite end; the palisade layer was depleted last. The acidity produced by the complete hydrolysis of the fat per bean is equivalent to 1 cc. of 0.1 N acid. The small amount of lipase present and the fact that the fat decreased markedly by the fourth day indicate that the rate of fat hydrolysis was limited greatly by the amount of active lipase present. On germination urease and protease increase during the first two days and then decrease, while lipase attains a maximum activity on the eleventh day and then decreases rapidly.

The data of FISCHER (9) indicate that the proteases of peas, beans, and buckwheat increase in the leaves with age. This differs from the changes of urease found in the leaves examined by the writer in which there is a rapid increase in urease to maturity and then a decrease. In the root tip urease is highest in concentration in the region of cell division and decreases rapidly in the elongation region. Peptidase of the barley root tip, however, is highest (18) in concentration in the region of cell elongation and not in the region of cell division.

#### UREASE AND RESPIRATION

If urease, unlike protease and lipase, is a growth-bound enzyme it might be expected that the changes in respiratory activity would normally parallel

the changes in urease activity, and that both of these factors would parallel, and be a measure of growth changes. A comparison of the data on the respiratory activity of legumes, as found in the literature, with the data on urease activity of parenchymatous tissue as here presented confirmed the suggestion that urease and respiratory activity run parallel. BEZAGU (2) working with leaves of various ages, of such species as *Robinia pseudoacacia*, *Cercis siliquastrum*, *Pinus sylvestris*, *Cobaea scandens*, *Ligustrum vulgare*, *Althaea*, and *Lovoglossum hercinum* found that the following facts were general for the respiratory activity of these leaves.

1. The amount of  $\text{CO}_2$  produced per gram of fresh weight per hour is highest in the youngest leaves and continues to decrease with the development and aging of the leaves in a manner similar to curve A, figure 5.

2. The  $\text{CO}_2$  production per leaf-hour is lowest for the very young leaves, increases rapidly to a maximum during elongation, and then gradually decreases as the leaf passes through the stages of maturity and senescence. These changes may be represented by a curve similar to B, figure 5.

This relation of respiratory activity to urease activity, that is, to the fact that urease is a growth-bound enzyme, is understandable upon the basis of the following assumptions:

1. Urease, in its active state, is a soluble protein which undergoes changes similar to other proteins in synthesis and decomposition.

2. The amount of soluble proteins (as distinguished from ergastic proteins), including urease, is a measure of the amount and activity of protoplasm.

3. Respiratory activity is related to protoplasmic activity or to the amount of catalytic (protein) surface upon which oxidation-reduction reactions take place.

#### UREASE AND PROTOPLASM

Much of the framework of protoplasm (23) is considered to be made up of proteins which are dispersed in an aqueous medium. The only measure of protoplasmic activity which is generally recognized is respiratory activity. Since urease forms a portion of the protoplasmic framework, it is suggested that the amount of protoplasmic framework will be proportional to the amount of active urease and to the respiratory activity. If these assumptions are correct, then the changes in protoplasmic framework or protoplasmic activity in a cell may be studied by observing the changes in urease activity under varying environmental conditions. In other words, the enzyme urease may be used as a "tracer." Although changes in respiration can be much more readily followed than urease changes in the study of protoplasmic activity, they have the disadvantage of being much more complex summation effects of numerous enzymic actions and various substrate concentrations than are the urease changes.

In the young embryonic cell there is a dense protoplasm which, as the cell elongates, later contains a number of vacuoles. With increasing age of the cell, the vacuoles combine to form a single large sap cavity so that the protoplasm becomes spread in a thin film along the cell wall. The data on respiratory and urease activity of leaf parenchyma cells are in accord, not with the interpretation that the protoplasm of the embryonic cell is merely stretched and diluted but that the total amount of protoplasm, or the protoplasmic activity per cell, actually continues to increase until the cell has attained its maximum size; after that, it decreases to a certain level as the cell ages.

#### UREASE AND PHYSIOLOGICAL AGE

As was noted in the first paper of this series (10), it is only the enzyme in a dispersed state, in which its active groups are available, which can be quantitatively determined. An interpretation of the curve of urease changes with age of the cell is therefore difficult to make. It seems certain, however, that the ascending portion of curve B figure 5, illustrating the increase of urease as the cell enlarges, can be interpreted in no other way than as a synthesis of the enzyme. It has been suggested as a possibility that an enzyme might not be synthesized *in situ* but might be transported as such to the growing regions. For example, the 10,000-fold increase of urease content from the youngest to the oldest seed of soy bean might be due to a transport of urease to the developing seed. The studies on urease indicate that the enzyme is not transported as such to other cells, for the following reasons: (1) Urease has not been detected in the secondary phloem tissue. (2) The disappearance of urease from the storage parenchyma cells of a cotyledon bears no observable relation to the proximity of these cells to fibrovascular bundles in their neighborhood. (3) When jack bean cotyledons, which contain the highest recorded amounts of urease, are kept in tap or distilled water for several days, the water will give no test for the presence of urease. These results may be due to the fact that this enzyme, being a protein molecule, cannot diffuse to any extent through the plasma membrane. It is interesting to note, in this connection, BACH's report (1), that urease is present in *Aspergillus niger* as an endocellular enzyme and that no urease is excreted into the culture medium.

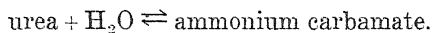
A satisfactory interpretation of the descending portion of curve B figure 5 must await a better understanding of the processes leading to senescence and death of a cell. It is difficult to evaluate the significance of the decrease in urease activity after the cell has reached the end of primary growth since the active enzyme may disappear because it is being catabolized by proteolytic ferments, or because it is being stored in a zymogen form, or because it is being inactivated. There is some evidence that catabolism of urease is

the major cause of the descending portion of the urease curve. From the work of SCHULZE and SCHÜTZ (22) and others it is known that leaves increase in protein N as they enlarge, and that during midsummer the content of protein N per leaf then begins to decline. This decrease in protein and in urease in the mature and aging leaves may perhaps be related to the fact that with increasing age of the leaf there is an increase in protease activity in the leaf (9). Peculiarly enough, the changes in urease and protease of the higher plants appear to run parallel with those found for *Aspergillus niger*. BACH (1) found an increase of urease in *Aspergillus*, the urease content reaching a maximum during the first 5 to 7 days and then rapidly declining to about one-sixth of the maximum value by the fifteenth day. This decline in urease content likewise coincided with an increase in proteolytic activity of the fungus. It is hoped in a future paper to deal with the question of the "element constant of protein," as related to the age and carbohydrate content of the cell, and its relation to urease activity.

#### RÔLE OF UREASE IN INTERMEDIARY METABOLISM

Since urease appears to be present in almost all plants and certainly to be present in all the organs of the jack bean and soy bean, what rôle does this enzyme play in the metabolism of plants? As is well known, an enzyme catalyzes the attainment of the equilibrium of a chemical reaction (4). Whether and to what extent hydrolysis of urea will take place will depend on the equilibrium constant of the reaction and on the presence of urea and water. The synthesis of urea will likewise depend on the equilibrium constant of the reaction and on the presence of ammonium carbamate.

It has been firmly established by MACK and VILLARS (19) and others that urease catalyzes the following reaction:



The forward reaction, that is, the hydrolysis of urea to ammonium carbamate proceeds very rapidly. It can readily be calculated<sup>5</sup> that about 2000 urea molecules make effective collisions per second with one urease molecule. The decrease in free energy of this hydrolysis is sufficiently great that urea is almost completely transformed into ammonium carbamate.

That urea can be absorbed by a legume plant from a sterile culture medium and can be split very rapidly into ammonia by the urease present in the cells has been reported by KLEIN (13). He has also shown that if large amounts of urea are fed, the ammonia produced becomes so great that signs of ammonia poisoning appear and the plant turns brown and dies. If one stops the feeding of urea, the total urea taken up disappears in a very short time.

<sup>5</sup> Assuming a molecular weight of 150,000 for crystalline urease; an activity of 26,000 U.U. per gram of crystalline urease (24); one active spot per urease molecule; excess of substrate; and that the reverse reaction is negligible.

The question of the presence in the plant of the substrate, urea, may be considered briefly. The investigations of KLEIN and TAUBÖCK (14) indicate that little free urea is present in the higher plants. The "urea" reported by previous workers was a complex of ureides which are apparently widely distributed throughout the plant kingdom. The ureides of both the higher plants and the fungi are split by boiling with dilute acetic acid; boiling with dilute alcohol does not decompose them. The ureides of higher plants are readily soluble in alcohol; the ureides of fungi are not. They report only two ureides in higher plants: the formaldehyde compound, which is found only in green assimilating plants; and the acetaldehyde compound which is formed in green unlighted or in chlorophyll-free organs as a result of respiratory processes. FOSSE believes that urea is present in combination with glyoxylic acid as allantoic acid. These stable ureide bodies remain in the organs for some time but at certain definite periods are attacked and disappear. Since the enzyme urease is present in the plant, it may be concluded that any free urea, released from the ureide combinations, from arginine, canavanine, purines, guanidine, etc., will be rapidly decomposed to liberate ammonia. The presence of large quantities of urease in the meristematic regions of the jack bean and soy bean suggests that urease performs a "necessary" function in releasing ammonia for protein synthesis.

Concerning the synthesis of urea by urease it has been postulated by ONSLOW (21), EULER (7), and others, that urea might be formed by the action of urease on ammonium carbamate. It has been realized, however, that when urea is brought into contact with urease an almost complete conversion of urea to ammonia results. But there remained the possibility that synthesis could occur in the cell if the cell had some means of removing from the field of enzyme action the small amounts of urea formed. For example, the trace of urea might be used to form a ureide, and then more urea would be produced because of the equilibrium involved.

It is possible to arrive at some definite conclusions as to the significance of urease in the synthesis of urea by making use of the data of LEWIS and BURROWS (16) on the hydrolysis of urea, and on the conversion of ammonium cyanate to urea (17). If urea, ammonium cyanate, ammonium carbamate, ammonium carbonate, or their ionic products are brought into water, an equilibrium will finally be established in which all of these substances will be present in certain definite proportions. For example, it may be calculated, that starting with a 1 M. urea solution in a closed container at 25° C. the products at equilibrium will be approximately 0.998 M. ammonium carbamate-carbonate, 0.002 M. urea, and a trace of cyanate.

Likewise, the maximum concentration of urea that may be synthesized by urease may be calculated. The highest amount of ammonia nitrogen in the jack bean, equivalent to 0.1 M.  $\text{NH}_3$ , was found in the radicle of a

16-day-old seedling (14). Assuming that sufficient  $\text{CO}_2$  is present to combine with all the ammonia present, then at this ammonia concentration approximately 80 per cent. of the ammonia will be in the form of ammonium carbonate and the other 20 per cent. will be in the form of ammonium carbamate, in a slightly alkaline medium (8). If this plant tissue is assumed to be neutral, the maximum theoretical amount of urea at equilibrium with this concentration of ammonium carbamate will be  $1.8 \times 10^{-6}$  gm. per gram of tissue. Even though the urea thus formed is slight, it might be removed from the sphere of urease action possibly to form a ureide and thus permit further urea synthesis.

Ammonium carbamate is, however, unstable on the acid side of neutrality and is very rapidly converted to carbonate. No ammonium carbamate is formed from ammonium carbonate in plants because plant tissues have a hydrogen-ion concentration that is generally on the acid side of neutrality. Since the enzyme urease catalyzes the reaction of ammonium carbamate to urea and water, no urea could be synthesized because no ammonium carbamate would be present; and no ureides would be formed. It may therefore be concluded that urease in the plant does not function in the synthesis of urea.

From the data on free energy change it becomes evident that no matter what steps are postulated for the synthesis of urea from ammonia and carbonic acid as they exist in the plant, some energy source, such as a coupled oxidation, is required (15). It is interesting to note that asparagine, another storage molecule for ammonia, is, like urea, an amide and also requires a coupled oxidation process to supply the energy for its synthesis (20). Whether urea formation by synthesis from ammonia is to be considered an important factor in explaining the significant quantities of ureides present in plants remains to be seen. The investigations of FOSSE suggested to KREBS (15) that "urea formation in plants follows mainly the same path as urea formation from purines in amphibians" that is, through the hydrolytic formation of urea from ureides rather than through a direct synthesis from ammonia.

The interesting paper by FEARON<sup>6</sup> ought to be mentioned. FEARON advances the hypothesis that cyanic acid is in some way responsible for urea formation. He believes that carbamic acid may form cyanic acid which together with ammonia will produce urea.

Insufficient attention has been paid by many investigators of hydrolytic enzymes to the energy values and equilibria involved in hydrolysis. LEWIS and BURROWS (16) established the fact that the free energy value in aqueous solution at room temperature of ammonium cyanate is greater than that of

<sup>6</sup> FEARON, W. R. The structure of urea with reference to its deamination and synthesis by urease. *Biochem. Jour.* 30: 1652-1660. 1936.

urea; likewise the free energy value of urea is greater than that of ammonium carbamate: *i.e.*, ammonium cyanate  $\rightarrow$  urea  $\rightarrow$  ammonium carbamate. If one assumes a 1 M. urea solution at room temperature and that no decomposition takes place to ammonium carbamate, it can be calculated that at equilibrium there will be 0.0067 M. ammonium cyanate and 0.9933 M. urea. But since urea is decomposing to ammonium carbamate, at equilibrium little or no cyanate will be present.

Any hypothesis which assumes that carbamate produces cyanate which in turn produces urea must in addition postulate an energy source for the synthesis of cyanate from carbamate.

### Summary

1. The urease activity of *Soja max* has been determined by the use of histological and quantitative methods. The data for total urease are summarized in the graph (fig. 4), which shows the total urease content of the developing bean, and of the soy bean seedling throughout the life history of the plant. The distribution of urease in the various tissues of seedlings 8, 15, 31, and 52 days old is illustrated in a composite picture (figs. 1, 2). A description of the specific urease changes of the individual plant structures is given.

2. In the soy bean, as in the jack bean, the changes observed in urease content of the plant are due primarily to the changes in urease content of the parenchyma cells. Neither the cambium nor the cells derived from the cambium contain amounts of urease that can be detected with the indicator reagents.

3. The changes in urease distribution found in the jack bean and soy bean may be considered to hold for the Papilionaceae in general.

4. Analyses of urease, lipase, and protease in the developing soy bean indicate that the changes in lipase and protease activities do not follow the changes in urease activity.

✓5. Urease is considered a growth-bound enzyme. There is a rapid synthesis of urease in actively dividing cells. Synthesis continues during the stage of cell elongation, at the end of which period the urease content of the cell reaches a maximum. The urease content of the cell then begins to decrease to a certain level.

6. The changes in respiratory activity and urease activity at various stages of cell development follow similar courses.

7. It is suggested that by observing the changes of urease activity one may obtain an indication of the changes in the protoplasmic framework and soluble protein content of a cell.

8. The data on urease and respiratory activity are in accord with the interpretation that protoplasmic activity in the parenchyma cell continues



to increase until the cell has attained its maximum size; after that it decreases to a certain level as the cell becomes older.

9. The rôle of urease in the intermediary metabolism of the plant is discussed. From thermodynamic considerations it is calculated that any urea present in the plant cell will be converted by urease into ammonium carbamate. Ammonium carbamate, being unstable on the acid side of neutrality (the condition in the plant cell), will be hydrolyzed to ammonium carbonate. It is also shown that urease cannot function in the synthesis of urea from ammonium carbonate in the plant cell. The presence of large quantities of urease, especially in the meristematic or protein synthesizing regions of the jack bean and soy bean, suggests that urease performs the function of releasing ammonia from any free urea present and thus makes it available for protein synthesis.

10. There is no evidence that urease is transported, as such, from one portion of a plant to another.

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UNIVERSITY OF MICHIGAN  
ANN ARBOR, MICHIGAN

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# SPECIFIC FACTORS OTHER THAN AUXIN AFFECTING GROWTH AND ROOT FORMATION

F. W. WENT

(WITH SIX FIGURES)

## Introduction

One of the most startling discoveries in the field of plant growth hormones is that auxin affects not only the growth in length of stems, petioles, etc., but also inhibits lateral buds, induces root formation, swellings, cambial activity, etc. This led to the conception that the auxins were stimulating substances without a clearly defined physiological activity or specificity (FITTING, 5). In a recent paper (WENT, 27) it has been pointed out that all effects of auxin other than cell elongation must be called secondary and that they all can be accounted for if we assume that other specific substances are activated in one way or another by auxin. A few experimental results pointing toward such an interpretation were offered but in the following paper the experimental data are given in full. It was endeavored to show that only in combination with other specific growth factors auxin influences root formation, bud growth, or swellings. It also gives an example of a new type of experimentation which has become possible through the availability of crystalline auxins. Whenever a plant or a part of a plant is flooded with auxin, other factors necessary for growth and development will become limiting, and in this way the existence of such factors can be demonstrated, and their concentration measured. The effects of the unphysiologically high auxin concentrations, however, must not be taken to mean that the naturally occurring auxin acts in the same way in the normal plant, as has been done recently (HITCHCOCK and ZIMMERMAN, 9). In the following paper auxin is interpreted only as a reactant, not as a factor in normal development.

The auxin used in all experiments to be described is indole-3-acetic acid, the so-called "hetero-auxin." The peas are a pure line of Alaska obtained through the courtesy of Dr. W. BROTHERTON. All experiments are carried out in a darkroom with controlled temperature (24° C.) and humidity (85 per cent). In darkness growth is simpler to interpret because synthesis of a number of substances essential or accessory for growth will not take place, and we need to consider only those already present or synthesized in darkness.

## Stem and bud growth

If we cut off the cotyledons from peas germinating in darkness, when the epicotyl has reached a length of one or a few centimeters, while the roots are

left in water, the growth of the shoot stops very soon. This is due to a lack of nutrients, because if a 2 per cent. sucrose solution is offered to the cut surface left by the removal of the cotyledons, growth is resumed and continues for a considerable time, at least for one week. Addition of auxin to the sugar solution has no effect. The same effect is produced when the roots are immersed in the sugar solution. This proves that the uptake of the sugar, necessary for growth, can take place either through the roots or through a cut surface. At least the roots are not necessary for the sugar uptake.

These results seemed, however, to contradict those obtained in the experiments on root formation, in which a piece of stem with an apical bud was cut off at the base and placed in sugar solution. The growing region of the stem in this case practically stopped elongating. From a number of other experiments, however, it appeared that for this growth in length of the stem *both* sugar and the presence of roots are necessary. To substantiate this statement two experiments are summarized in table I. Peas are germinated on wet filter paper in the darkroom. As soon as the shoots are about 1 cm. long they are transferred to small bottles, one per bottle, into which a few cc. of water are poured. When the plants have reached a suitable length (about 10 cm.) they are divided into four equal groups. One group is left intact, with both roots and cotyledons; in the second group the roots are cut off just below the cotyledons, which are left; in the third group the cotyledons are cut off close to the stem, leaving the roots; and in the fourth group the roots with cotyledons are cut off just above the insertion of the latter. In the first and second group the seed coats are removed to prevent premature decay. Then each bottle is filled with 5 cc. of 2 per cent. pure sucrose.

The growth of the four groups is summarized in table I; the results are clear-cut and are borne out by a number of other experiments not recorded here. In the first experiment the growth has been recorded for the first four days after the operation only: the growth of the plants without either roots or cotyledons is intermediate between those with or without both. In both groups lacking roots the growth drops off much more rapidly in the third and fourth day than in the groups with roots. The same is seen in the second experiment, where the growth has been followed for nine days: in the last period of four days the growth of the plants without cotyledons but with roots is even twice that of those without roots but with cotyledons, so that the former actually overtook the latter in length. This second experiment is also shown in figure 1, where the relatively constant growth rate of both groups with roots stands out.

When both roots and cotyledons are cut off the shoots practically cease growth. Only during the first two days is there a small residual growth.

TABLE I

TOTAL GROWTH AND GROWTH RATE OF ETIOLATED SHOOTS OF PEA SEEDLINGS AFTER COTYLEDONS OR ROOTS OR BOTH HAVE BEEN CUT OFF.  
LENGTH OF SHOOTS AT BEGINNING OF EXPERIMENT 10-15 CM. EACH FIGURE IS THE MEAN OF 10 PLANTS

CONDITION OF PLANTS	EXPERIMENT 1			EXPERIMENT 2				
	GROWTH IN 4 DAYS	GROWTH RATE IN MM./DAY FOR PERIOD		GROWTH IN 9 DAYS	GROWTH RATE IN MM./DAY FOR PERIOD			
		1-2	3-4		mm.	mm./day	mm./day	mm./day
With cotyledons with roots.....	101	26.5	24.1	206.5	32.0	29.7	13.3	
With " without roots..	48	15.1	9.0	80.8	16.9	10.9	3.6	
Without " with roots .....	38	10.0	9.0	91.9	15.2	10.9	7.2	
Without " without roots...	8	3.4	0.7	19.0	5.2	1.4	1.1	

The most outstanding fact seems to be that plants with cotyledons, but without roots, having an abundant food and sugar supply, are far behind in their growth compared with normal plants. This result is not changed if the plants are grown under a bell jar in an atmosphere saturated with water vapor, indicating that the absence of the roots does not primarily interfere with the water uptake. Whereas in the course of a week the growth of the groups without roots but with cotyledons, and those without cotyledons but with roots, has reached about the same total, the type of growth is completely

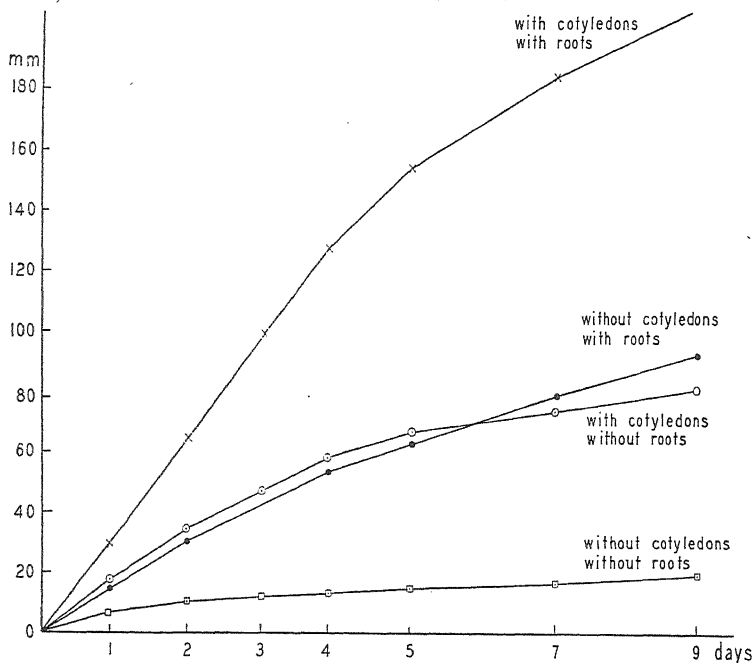


FIG. 1. Increase in length (ordinate in mm. beyond the original length of 100–150 mm.) of etiolated pea seedlings, after removal of roots or cotyledons. Abscissa: days after operation. Each line represents the mean of 10 plants.

different. In the first case the growth drops off rather rapidly, indicating a rapid depletion of the factor necessary for growth in length, whereas in the second case it is evident that this factor is continuously supplied. Thus we reach the conclusion that the roots form, and the cotyledons store to some extent, a factor required for growth in length of the stems. There is no doubt about the necessity of auxin for the elongation of the stem, so that the factor coming from the roots has to be considered as a second growth factor, which has to cooperate with auxin to make growth possible. A lack of either will stop stem elongation. It is very likely that this factor is one of those summarized under the name “food factor” (WENT, 23, 25).

A question arises about the nature of this factor coming from the roots. It might be that the roots are able to concentrate sugar and whatever salts may be present in the sugar solution. This is not likely, because as shown in the beginning of this section the effect of the roots is apparent only when sugar is present, and this sugar may be taken up through the cut cotyledonary petiole.

To obtain further evidence a number of grafting experiments have been performed. The same etiolated pea seedlings are used. When the stem has reached a length of about 10 cm. it is cut off just below the first node. Then

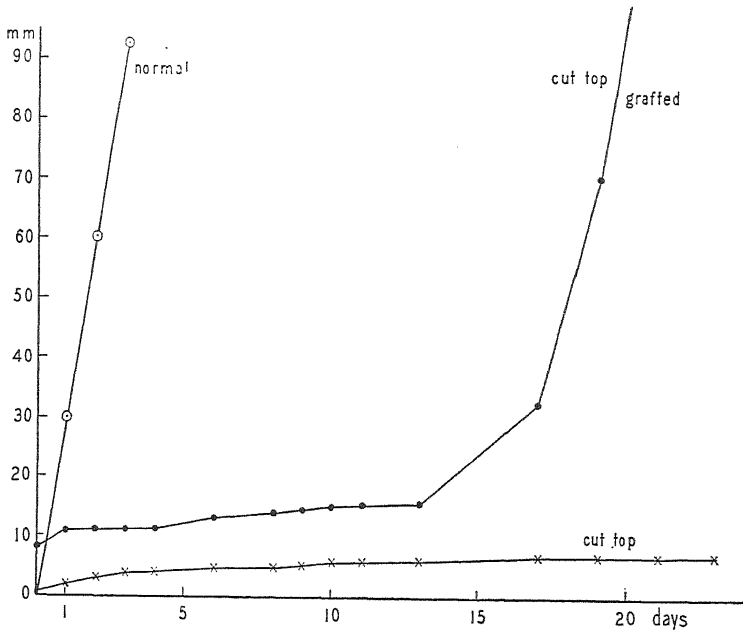


FIG. 2. Increase in length (ordinate represents mm. growth beyond the original length of 100–150 mm.) of etiolated pea seedling stems, in the course of 23 days after operation (abscissa). Circles: growth of normal untreated plants. Crosses: growth of shoots cut above the cotyledons. Black dots: growth of cut shoots, grafted on their roots + cotyledons.

it is replaced, sealed with gelatin or agar, and held in place by a short piece of glass tubing with a bore of the same diameter as the stem. Although the gelatin eventually becomes liquified by bacterial activity it still gives distinctly better results than agar. Recently still better results have been obtained by direct contact of the tissues without the use of gelatin or agar. The growth of the grafted stems is then measured for a period exceeding ten days. During the first two days some residual growth still takes place just as in the experiments in which cut stems are placed in sugar solution.

Growth then ceases completely, as will be seen from figure 2. After one or two weeks a certain number of the grafted stems suddenly resume growth and develop normally, at practically the same rate as not-operated seedlings. It has been found that in all those cases where growth is resumed a fusion of the tissues of graft and stock has occurred, and that this fusion is still lacking in the others which do not grow. The fusion takes place between the vascular tissues. This experiment, which has often been repeated, shows that the factor, coming from the roots and necessary for the growth of the shoots, does not pass a cut surface but moves through living tissue only. Whether this is due to inactivation of the active substance outside the cells or otherwise, has not been determined as yet. For the argument, however, this is not essential. Thus these grafting experiments confirm the conclusion, that a special substance is formed in the roots, which moves upwards from cell to cell towards the apical parts of the pea stems where it causes growth in length of the shoot in conjunction with auxin. I propose to call this substance *caulocaline*.

This factor coming from the roots is the same as the factor necessary for growth of the axillary buds, as shown by the following experiments. If the same four groups of peas are made: one with both cotyledons and roots, one with cotyledons but without roots, one with roots but without cotyledons, and one without either roots or cotyledons, and if these plants are then placed in 2 per cent. sugar solution and decapitated, only the first three groups will develop axillary buds, as shown in table II. In these experi-

TABLE II

GROWTH OF AXILLARY BUDS IN PEA PLANTS DECAPITATED IMMEDIATELY AFTER REMOVAL OF ROOTS OR COTYLEDONS. EACH FIGURE IS THE MEAN OF 10 PLANTS

CONDITION OF PLANTS	EXPERIMENT 1		EXPERIMENT 2	
	GROWTH OF AXILLARY BUDS		GROWTH OF AXILLARY BUDS	
	WITH AUXIN	WITHOUT AUXIN	WITH AUXIN	WITHOUT AUXIN
With cotyledons with roots .....	7.1	65.8	8.3	26.3
With       "       without roots	3.6	14.0	1.0	1.9
Without   "       with roots .....	5.4	22.7	3.9	21.2
Without   "       without roots	1.0	1.0	1.0	1.0

ments the effect of the roots is still more pronounced: with roots but without cotyledons the growth is almost twice that of the plants without roots but with cotyledons. This difference from the experiments on the growth of the main shoots (table I) is easily explained and even to be expected, for we have seen already that the effect of the cotyledons is greatest in the first days,



and then drops off rapidly because of a depletion of the caulocaline-factor in the cotyledons. In the plants with roots there is a continuous supply of this factor, even after a week. The axillary buds start to grow rapidly only four or more days after decapitation, when the plants with cotyledons but without roots are low in the growth factor coming from the roots. Table II also offers a good example of bud inhibition by auxin. In the columns "with auxin" the plants are treated immediately after decapitation with a concentrated auxin paste. This delays the bud growth, but does not otherwise change the results. This must be interpreted to mean that auxin inhibits lateral buds *through* this other factor necessary for bud growth, caulocaline.

Another remarkable and consistent effect has been noticed in all these experiments. In the plants with cotyledons but without roots, just as in those with both, only the buds in the axil of the upper scale develop, whereas in the plants without cotyledons but with roots the buds on the lower node or the cotyledonary buds (which are not removed with the cotyledons) grow out. The explanation has to be sought in the relative concentrations of auxin and caulocaline. In all cases the buds present start to grow out, but soon the lower or the upper overtakes and then inhibits the others. With an abundant auxin supply (when cotyledons are present) the upper bud inhibits the lower through its auxin production. Without much auxin (cotyledon cut) the upper buds do not inhibit the lower ones; as the latter are closer to the caulocaline supply they will grow better.

### Swellings

The growth of stems stops soon after decapitation because of lack of auxin. Growth stops after cutting the stem at the base because caulocaline becomes limiting. Addition of auxin to the apical cut end of a decapitated pea stem has an effect on elongation only in the lowest concentrations. Slightly higher auxin concentrations (in contrast with the *Avena* coleoptile) inhibit growth in length and still more auxin causes the upper few mm. of the decapitated stem to swell. Thus there is a continuous series of concentrations giving first elongation, then growth inhibition and swellings. The following experiments make it probable that not only for growth in length but also for swellings the cooperation with auxin of a factor coming from the roots is necessary.

Again the same four series of plants are prepared and placed in sugar solution. They are decapitated and a concentrated auxin paste (1 part indole-3-acetic acid in 500 parts lanolin) is applied. In another series the decapitation is carried out two or three days after removal of the roots or cotyledons. At that time the plants should have used up whatever growth factors they had at the moment of de-rooting, and only in those plants with a continuous supply should swellings occur. Table III summarizes the re-

TABLE III

DIAMETER OF APICAL SWELLINGS OF PEA STEMS DECAPITATED 5 MM. BELOW TIP AND TREATED WITH CONCENTRATED AUXIN PASTE. DECAPITATION AND APPLICATION OF AUXIN EITHER IMMEDIATELY AFTER CUTTING OF COTYLEDONS AND ROOTS, OR TWO DAYS LATER. EACH FIGURE IS THE MEAN OF 10 PLANTS

CONDITION OF PLANTS	EXPERIMENT 1		EXPERIMENT 2	
	SWELLINGS WHEN		SWELLINGS WHEN	
	AUXIN ON IMMEDIATELY	AUXIN ON AFTER 2 DAYS	AUXIN ON IMMEDIATELY	AUXIN ON AFTER 2 DAYS
	mm.	mm.	mm.	mm.
With cotyledons with roots .....	4.4	4.2	5.5	4.6
With " without roots	4.0	2.8	5.0	2.5
Without " with roots .....	4.4	3.6	4.0	3.6
Without " without roots	3.7	2.4	3.6	2.4
Stem diameter before treatment	2.2	2.2	2.2	2.2

sults of two such experiments. It will be seen that auxin by itself is not able to produce appreciable swellings. Only in cooperation with something coming from the roots do the swellings appear. Again this factor moves through living tissue only, as evidenced by the following experiment. Many pea stems are cut off and sealed on their respective root systems (with cotyledons) again. At set intervals the length of the shoots is measured, and some of them are decapitated and strong auxin paste is applied. Only in those plants in which the growth of the shoots has been resumed (indicating the successful junction of the tissues) appreciable auxin swellings appear. Although the direct experiment still stands out there is strong evidence that the same factor coming from the roots is necessary to give growth by cell elongation—in conjunction with low auxin concentrations—or will give swellings with excess auxin. The probable relation between such swelling and growth in length has been discussed elsewhere (WENT, 27).

CZAJA (4) has observed that when the cotyledons are removed from *Vicia faba* seedlings no swellings occur after auxin treatment. From the picture he publishes it is clear, however, that his effect may have been the result of a lack of roots rather than lack of cotyledons, as he concludes. Based on their own experiments JOST and REISS (1936) conclude that removal of cotyledons does not prevent the formation of auxin swellings on pea epicotyls. Since we know that auxin effects (like bud inhibition) may be transmitted across a cut surface even without actual junction of the tissues of the graft (SNOW, 19), the experiments mentioned above with grafted pea stems form another argument against the hypothesis of CZAJA (3) that the swellings occur as the result of two streams of auxin, one coming from the base.

### Leaf growth

It still might be argued that in all the preceding cases the roots contain a certain amount of food necessary for growth and development in general, or that they are able to transform sugar into such food, so that every growth

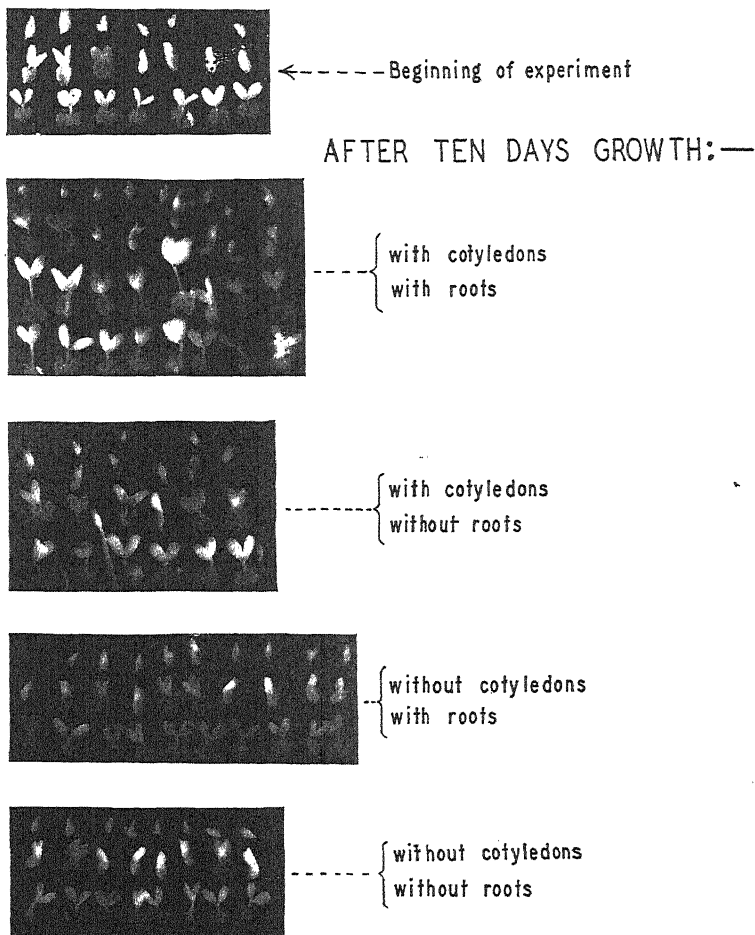


FIG. 3. Shadow photographs of all leaves with petioles and stipules of etiolated pea plants in one experiment. Leaves + terminal bud of each plant are cut off and arranged in a vertical row. Above, leaves of a set of plants at the moment the cotyledons or roots were cut off the others.

process will be speeded up by the presence of roots. This is by no means the case. The effect of the roots is of a more special nature. For if we inspect the plants used for the experiments summarized in table I, a very marked

difference will be observed in the size of the terminal buds, leaves, and stipules, which differences do not go parallel with the presence or absence of roots. Figure 3 shows shadow pictures of all the leaves which have developed on the different groups of plants, and also the size of the leaves on a set of plants just before treatment, which can be considered as the controls. Table IV contains the actual measurements taken from these pictures.

TABLE IV

MEASUREMENTS OF LEAF DIMENSIONS OF THE EXPERIMENT FROM FIGURE 3 TEN DAYS AFTER REMOVAL OF EITHER COTYLEDONS OR ROOTS

CONDITION OF PLANTS	MEAN LEAF SURFACE			PETIOLE AND LEAF LENGTH				
	1ST LEAF	2ND LEAF	TOTAL	1ST	2ND	3RD	4TH	TOTAL
	mm. <sup>2</sup>	mm. <sup>2</sup>	mm. <sup>2</sup>	mm.	mm.	mm.	mm.	mm.
With cotyledons with roots .....	17.7	24.5	42	12.8	14.3	10.2	4.6	41.9
With cotyledons without roots .....	22.0	19.5	41	13.2	11.3	6.6	3.1	34.2
Without cotyledons with roots .....	14.3	± 10	24	9.8	8.5	5.2	2	25.5
Without cotyledons without roots .....	13.8	± 10	24	10.3	8.4	4.6	2	25.3
Before treatment .....	13.8	± 10	24	9.4	8.3	5.0	2	24.7

Where the cotyledons are cut off neither leaves nor stipules increase in size, even in presence of the roots. On the other hand, absence of the roots does not prevent the leaves from developing, provided the cotyledons are left, in which case they almost double their leaf surface in 10 days. The same relationship is found if we measure the length of leaf blade plus petiole: no increase in length occurs if the cotyledons are absent. This result has been confirmed in another experiment; indeed, in all experiments reported here this phenomenon is met with. It accounts for the curious shape of the stems growing in sugar solution with their cotyledons cut off. During normal growth the terminal bud continues to increase in size by the successive development and growth of new leaves, so that when a new internode elongates and the leaf at its lower node does not envelop the bud any more, this bud has grown and has the same appearance as the bud in the younger plant. But when the cotyledons are cut, neither the terminal bud nor its enveloping leaves grow any more, so that when through growth of the stem the successive internodes develop and peel off the preformed leaf primordia from the terminal bud, the latter seems to shrink until it is not more than an insignificant appendage, and such a stem looks almost pointed. The same thing happens with the growth of the axillary buds: when only roots are present

they develop into long spear-like shoots, whereas the lateral buds on plants with cotyledons are topped by a fair-sized bud.

If etiolated pea plants, even without cotyledons, are placed in light of sufficient intensity, the stipules and leaf blades will develop and grow considerably. This necessity of light for leaf blade growth is well known, and the lack of leaf development in darkness is one of the characteristics of the phenomenon of etiolation. In KOSTYTCHEW-WENT (11) a good example of the effect of light of even short duration on the growth of *Vicia faba* leaves is depicted (p. 294). GREGORY (6) has quantitatively measured the increment in leaf surface of *Cucumis* plants under controlled conditions, and concludes that a special factor, necessary for leaf growth, is formed in the older leaves under the influence of light. This factor is not directly related with photosynthesis of carbohydrates, since the  $Q_{10}$  of photosynthesis is very different from that of leaf growth.

I observed a curious case of deficient leaf growth in extreme shade in the Botanic Gardens in Buitenzorg, Java. *Gonocaryum* grows there in the shade as a small tree. Owing to its large thick leaves it casts a dense shade on the ground so that only a few of the most pronounced ombrophilous plants can grow there. The fruits of this tree are large and enclose only one seed, which contains an excessive amount of storage food. The germination of a fruit, lying under the tree, is normal, and a shoot with many normal leaves develops. The shade, however, is so dense that soon these leaves are shed and the growth of the main stem stops. Axillary buds then develop, the first of these shoots still bearing some leaves, although their leaf blades are small and mottled. Apparently the reserves of leaf-growth substances in the seed are exhausted long before the rest of the storage food and the auxin is used up. Thus for many months such seedlings continue to send up shoots, without leaves, but with very small scales which develop instead. If such a seedling with the appearance of a witches'-broom, and without a single leaf, is brought into full daylight, then at once new, though small, normal leaves start to develop instead of scales. This observation seems also to warrant the conclusion that in the light new leaf growth substances are formed.

The differentiation between various growth factors by removal of cotyledons, roots, apical buds, etc., is relatively rough; a much more elegant way is afforded by the use of monochromatic light. This at least is indicated by experiments which have been performed by growing cut pea shoots in different colored lights, obtained by filtering the light of a strong incandescent lamp through sets of Corning glass filters. Yellow and blue light seemed to favor leaf growth; red favors stipule development; bud growth was best in orange and yellow. Green light was practically ineffective for all processes studied; plants subjected to it behaved as if they were grown in darkness.

In the case of leaf-blade growth practically no evidence exists which links the activity of leaf-growth substances with auxin. Quite an amount of circumstantial evidence, however, points toward the conclusion that auxin specifically conditions petiole and vein growth in the leaf, that is, conditions growth of those tissues which are built up from the beginning by elongated cells. Experiments, described by LAIBACH (12) and AVERY (1), show that auxin after one-sided application causes bending of the midrib due to a growth increase on the side of the application. I have confirmed these experiments on a number of different plants and have reached the conclusion that auxin does not seem to increase mesophyll growth, although it increases vein growth. The same effect, but in opposite direction, has been described by MARESQUELLE (16). When leaves have been attacked by lice, the mesophyll bulges out without being affected otherwise, because only the growth of veins and midrib has been decreased as a result of the sucking of the aphids. Similarly there are some virus diseases known which specifically affect stem and vein growth, and in one such case GRIEVE (1936) has shown that this is correlated with a lack of auxin in the virus-attacked plant. In all these cases only vein growth is affected, and not the mesophyll. The opposite case: deficient mesophyll development without change in vein growth is found in certain hereditary deficiencies, "deformis" in tobacco (HONING, 10) and "wiry" in tomatoes (LESLEY and LESLEY, 15). In view of this indirect evidence it seems sufficiently established that the growth of a leaf is a complex phenomenon as far as the growth of its constituents is concerned. Auxin regulates midrib and vein growth, but does not influence mesophyll development, which is governed by a special leaf growth substance formed in leaves in the light and stored in pea cotyledons, which I propose to call *phyllocaline*.

### Root formation

In the preceding sections we have seen that apart from auxin another factor is needed for growth in length of pea stems, and that, when these stems have been cut off, this factor disappears from them within about two days. A similar (or identical) factor, necessary for the production of swellings by excess auxin, likewise disappears in the course of two days after cutting the stems.

In the case of root formation a slightly different situation might be expected, because to induce the initiation of roots we deliberately cut the pea stems near the base before we treat them with auxin. This might be interpreted by assuming that auxin by itself is sufficient to induce root formation. That this is not true will be shown in the following section.

In previous experiments on root formation the number of roots, visible to the naked eye near the basal cut surface of a pea stem fourteen days after

treatment, has been used as a measure for the effectiveness of the hormone treatment (WENT, 24). When treated with relatively low auxin concentrations in water, under the prescribed conditions, the number of roots formed is rather small and the number of visible roots approaches the total number initiated. In the present studies, however, excessively high auxin concentrations have been used (0.5–2 mg. of indole-3-acetic acid per gram of lanolin), and since the lanolin is left on the treated plants, the auxin action is continuous over a long period of time. Under these conditions only a small number (20 to 40 per cent.) of all roots initiated by the treatment grow out and become macroscopically visible. This is mainly due to two factors: in the first place, an excess of auxin prevents root elongation, and secondly, the material necessary for root growth in length is limited, so that an increase in number of roots initiated will cause a decrease in their ultimate size, until the majority never grow out. This means that, in the experiments to be described, the roots had to be counted under a microscope with the aid of cross sections of the treated pea stems. Since it was found that the middle part of such stems never form any root primordia, the upper and lower 5 mm. of each stem (or a longer region if necessary) have to be cut by hand into sections of about 0.1 to 0.2 mm. thickness, which are placed in order on slides. With a low power the root primordia are then easily visible at the transition between central cylinder and cortex, mostly alternating with the four primary vascular bundles of the central cylinder, or in connection with the four small cortical bundles. Care has to be taken not to count the same root twice if it appears in consecutive sections. Very close to the point of auxin application at the apical cut surface the root primordia occasionally seem to have fused into a wide meristematic plate, and then arbitrarily this has been (mentally) divided into a certain number of root primordia.

The first experiment to be described is directly comparable with the previous experiments on growth in length. Etiolated pea seedlings are divided into three groups: from one the roots are cut off, from another only the cotyledons are removed, and the plants from the third group are left intact. Each plant is placed in a separate bottle with a few cc. of 2 per cent. sucrose solution. After four, six, or seven days a few of the treated shoots are cut off just above the second scale and placed in 2 per cent. sucrose; the 5-mm. tip is removed and a concentrated auxin paste then applied to the apical cut surface. Seven days later the peas are sectioned and the number of roots initiated is determined. Table V summarizes the results. For each stem individually is recorded the number of roots formed near the apical and basal cut surface of the pea stems. The figures marked "total" give the total number of roots formed per plant per treatment. It seems that as the plants grow older root formation on stems drops somewhat. This

TABLE V

NUMBER OF MICROSCOPICALLY VISIBLE ROOTS AND ROOT PRIMORDIA FORMED ON CUT PEA STEMS. THEY HAVE BEEN CUT AND TREATED AT THEIR APEX WITH AUXIN PASTE 4, 6, OR 7 DAYS AFTER REMOVAL OF EITHER COTYLEDONS OR ROOTS

STEMS CUT FROM PLANTS	ROOTS FORMED	TIME BETWEEN REMOVAL OF ROOTS OR COTYLEDONS AND AUXIN APPLICATION					
		4 DAYS		6 DAYS		7 DAYS	
With cotyledons with roots	At apex .....	20		13		9	
	At base .....	8		7		7	
	Total .....		28		20		16
With cotyledons without roots	At apex .....	21		8		6	
	At base .....	8		4		5	
	Total .....		29		12		11
Without cotyledons with roots	At apex .....	10, 9, 3		2, 3		1, 2	
	At base .....	3, 3, 3		1, 0		2, 0	
	Total .....		10		3		2.5

decrease is only about half the original value of that in the presence of the cotyledons, but when the cotyledons have been removed root formation practically falls to zero. There is a great difference between the group with cotyledons but without roots, with a total average of 17 roots formed per plant, and the group with roots but without cotyledons with an average of only 5 roots. Both groups had about the same growth in length, so that here we have a clear case of two processes, both dependent upon auxin, which are independent of each other because of the intervention of other factors: root formation is conditioned by auxin and a factor supplied by the cotyledons, and growth in length takes place under the influence of both auxin and caulocaline.

The experiment described above makes it clear at once that root formation is not merely a function of the cell as such, even if it is supplied with sufficient auxin, but it is rather a function of the specific condition of the tissue, depending upon the presence of a specific factor coming from the cotyledons which cooperates with auxin. This conclusion is confirmed by the following set of three experiments. In each of them a number of long (30 to 40 cm.) etiolated pea shoots, grown under standard conditions, are selected for uniformity. These shoots are cut off just above the cotyledons and 5-mm. tips are removed. They are then either left intact or cut into 2, 3, 4, 6, 8, or 10 pieces. Each piece is placed with its base in 2 per cent. sugar solution, and all sections from one plant are either treated by applying auxin paste (1 part indole-3-acetic acid in 500 parts of lanolin) to the apical cut surface, or are not treated and left as controls. Nine days later both the



apical and basal portions of each piece are sectioned, and in figure 4 the actual number of roots counted in each case is listed. The vertical lines represent the pea stems; where these lines are interrupted the stems have been cut. Each number from 1 to 24 (top row) represents one stem; all figures below one stem number refer to roots formed on the sections of that stem. All numbers at the right of the upper part of a section are the roots formed

PLANT CUT INTO	1	2	3	4	8	6	10
No	1 2 3 4 5	6 7 8 9 10 11	12 13 14 15	16 17 18	19 20 21	22 23	24
	26 18 31 30 30 27	18 11 32 7 10 3 13	8 8 23 24 15	8 5 20 11	4 3 4 0 5 3	5 14 10	23 0
			19	13	6	13	13
					8	17	4
					7	20	18
					5	22	2
					6	26	3
					7	22	0
					8	18	18
					9	20	2
					10	22	3
					11	22	0
					12	20	18
					13	20	2
					14	11	10
					15	7	1
					16	16	6
					17	16	1
					18	10	5
					19	10	5
					20	10	5
					21	10	5
					22	10	5
					23	10	5
					24	10	5
TOTAL PER PLANT	35 31 37 30 34	41 38 46 43 19 11	55 53 33 52	66 33 27	47 39 43	38 75	172
Mean	7.4	3.3	5.0	4.2	4.3	8.1	
CONTROL	4	5	6	4	10	5	

FIG. 4. Root formation after apical auxin application, at apical and basal cut surface of sections of pea stems. The stems have prior to auxin treatment been cut into 1, 2, 3, 4, 6, 8, or 10 sections. To the right of each section are the number of roots formed at apex and base in the individual plants, and the means. To the left is the total mean.

near the apical cut surface; to the right of the lower end of each section are indicated the roots formed near the base. For convenience sake the means and totals of roots formed are calculated, per apical or basal end as well as per section (to the left of each line representing the section) and per plant (bottom row). Plant numbers 5, 10, 11, 17, 18, 20, and 21 belong to the first experiment; 1, 2, 6, 7, 12, 13, 16, and 19 to the second; and 3, 4, 8, 9, 14, 15, 22, 23, and 24 to the third. It will be seen that there is an enormous variation in the root formation per apex or base, although the totals per plant are

more constant. This variation is only partially due to the fact that the results of three individual experiments have been combined.

If we try to draw a conclusion from figure 4, then it is clear that one point stands out. If for a moment we disregard the plants 22, 23, and 24, it is clear that the number of roots formed per plant is the same irrespective of whether such a stem has been cut into 1, 2, 3, 4, or 8 pieces. This is not caused by a constant root formation at the apex of the apical section and at the base of the basal section, but the number of roots formed at the apex of the top section is roughly inversely proportional to the number of sections cut from the stem, indicating that the roots are formed at the expense of some factor distributed over the whole length of the stem. For this factor the name rhizocaline may be suggested, previously used for the root-forming factor moving downward in the stem which was found to be identical with auxin (WENT, 26). The maximal root formation on one stem only occurs when an excess of auxin is applied at the tip of each section. Similar sections, not treated with auxin, form only a few roots, and these only near the basal cut surface. The latter appear under the influence of the auxin present in the plant, and almost exclusively on sections bearing either a scale or a leaf with axillary bud. Table VI gives a summary of the influ-

TABLE VI  
ROOT FORMATION ON PEA STEM SECTIONS FROM LONG ETIOLATED PLANTS

ROOTS FORMED PER SECTION	SECTION COMPRISING				
	1ST SCALE	2ND SCALE	1ST LEAF	2ND LEAF	WITHOUT NODE
Controls, no auxin .....	4.0	2.3	1.1	0.8	0.0
Treated with auxin ...	5.5	8.0	9.6	14.8	10.1

ence of scale or leaf (or perhaps rather of their axillary buds) on root formation in sections treated or not treated with auxin. It shows that everywhere there is enough rhizocaline present to form a considerable number of roots after auxin treatment, independent of the presence or absence of buds, scales, or leaves. The number of roots formed in the controls decreases from the more basal towards the more apical nodes which has to be interpreted in terms of a decreasing auxin production from basal to apical lateral buds. THIMANN and SKOOG (21) found the opposite in *Vicia faba*, but their experiments were carried out with plants grown in the light. The presence of buds has in itself no influence on the distribution of roots over apical and basal cut surface of a section after auxin treatment. The ratio of roots at apex to roots at base is 3.5:1 when a leaf, 3.5:1 when a bud, and 4.0:1 when no node is present in the section.

Perhaps the most interesting conclusion, with far-reaching implications, to be drawn from figure 4 is the following. From the root formation on the 4th or 8th sections of the stem it is evident that the rhizocaline is present everywhere in the stem, but that its concentration drops towards the base of the stem. Now the root formation on the non-cut stems shows that all rhizocaline present in them becomes active at either tip or base, even that portion which was present in the middle sections of the stem. This leads to the conclusion that auxin causes a redistribution of the rhizocaline in the stem.

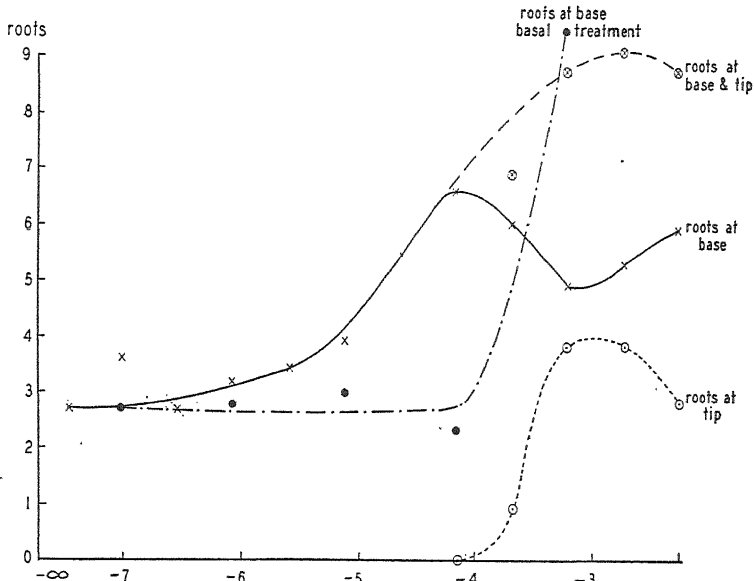


FIG. 5. Macroscopically visible roots (ordinate: roots formed per plant) on pea stems, 14 days after apical (circles and crosses) or basal (black dots) auxin treatment. Abseissa: log of the auxin concentration (in mol per liter).

The next experiments confirm this conclusion. Before considering them, however, we may examine for a moment the plants 22, 23, and 24 of figure 4, which show such a different behavior. Plants from the same series cut into 1, 2, and 3 sections formed a total of 36, 44, and 45 roots, behaving like the others in that the number of sections does not materially influence the total number of roots formed per stem. But those cut into 6 and 10 sections have formed 81 and 172 roots. It is impossible to give any satisfactory explanation for these exceptions to the scheme adopted; that we have to consider them as exceptions also follows from the following experiments.

Three hundred pea shoots, all about 15 cm. long, are cut above the first scale and 5 mm. below the terminal bud in the same way as plants are prepared in the standard test for root formation (WENT, 24). They are treated

for 14 hours with auxin solutions in water of varying concentration, either at the base or at the (split) tip. For six days they are left standing with their base in 2 per cent. sucrose solution, then transferred to tap water, and 14 days after the beginning of the experiment the number of macroscopically visible roots is counted, both at the tip and base. The results are reproduced in figure 5 (partly redrawn from WENT, 27). It is evident that after apical auxin treatment the number of roots formed near the base increases with increasing auxin concentration. Around  $10^{-4}$  molar concentration the maximal number of roots is formed; beyond that concentration it decreases again. At the same time, however, roots are formed near the apex; if the numbers of roots formed near base and apex are added together the curve representing the relation between auxin concentration and root formation rises smoothly towards a maximum of 9 roots per plant. Below  $10^{-4}$  molar the root formation caused by indole-3-acetic acid is perfectly polar, as in the normal plant. But above that concentration the polarity seems partly reversed. Exactly the same happens when the plants have been treated with auxin at their base. Below  $10^{-4}$  molar auxin basal treatment has no effect whatsoever on root formation, but immediately above this concentration the maximal number of roots (9.4 per plant) is formed near the base. At that concentration ( $10^{-3}$ ) the polar uptake of auxin seems to be abolished. A still higher concentration is highly toxic and thus does not cause any root formation at all. To compare this behavior with what happens inside the normal plant, we have to bear in mind that the auxin concentrations occurring in nature are of the order of  $10^{-6}$  molar or less. This means that in the normal plant, and also in plants treated with auxin concentrations up to 100 times normal, root formation is a perfectly polar phenomenon: apical auxin application induces root formation at the base, basal application does not have any effect at all. Auxin concentrations of  $10^{-4}$  molar or higher induce root formation at the place of application.

That the experiment of figure 5 is by no means an exception is shown by a number of earlier experiments which will not be considered in detail. In three experiments, each comprising about 200 plants, it was found that in order to cause root formation on pea stems by basal auxin treatment a 100–1000 times higher auxin concentration was required than for apical treatment. Numerous experiments have shown that when very concentrated auxin solutions are applied to the tip of pea cuttings, the number of roots formed at the base is less than with lower concentrations, and simultaneously roots appear near the treated tip. These roots are able to grow out, because the high auxin concentration acts only during the 15 hours of application, and the growth of the roots occurs later when the excess auxin has disappeared.

It is evident from these experiments that the roots at the apex are formed at the expense of those at the base. Here again we have a very clear

case of a developmental process being limited by a factor other than auxin, and we reach the same conclusion: namely, that a factor, which we will call rhizocaline, is present in pea stems in limited amounts. Without this factor no root formation is possible. Low auxin concentrations inside the stem make the rhizocaline move downwards—in the same direction as the polar auxin movement—and cause root formation at the base. At high auxin concentrations, above  $10^{-4}$  molar, roots will form at the place of auxin application, independent of the innate polarity of the stem.

### Discussion

If we survey the evidence collected in the foregoing experiments there are a few important points which should be stressed and considered in their relations to other known facts.

Four different processes in the development of a pea seedling have been studied: namely, growth in length of stems and lateral buds, apical auxin swellings, root formation, and leaf growth. The first three were known to depend on the presence of auxin, and since up to the present it has been principally the effects of auxin on these processes which have been studied the specificity of auxin has consequently been questioned. Thus the old idea of stimulus—which never had quite disappeared from the minds of the older generation of plant physiologists in relation to development—came up again. Thus, auxin should “stimulate” cells, and, according to the disposition of each cell, it should grow in length, swell, form roots, or do something else. In another paper (WENT, 27) this problem has been discussed in greater detail. Here it suffices to say again that the stimulus concept involves a setting free, or release, of the energy of a cell previously in a metastable equilibrium. This involves no stoichiometrical relationship between stimulus and reaction. Now one of the most striking properties of the auxins is their quantitative action; the more we come to know about them, the clearer this stands out. For a whole series of compounds (including indole-acetic, indole-propionic, indole-butyric, indole-valeric, naphthalene-acetic and anthracene-acetic acid) it has been found that per mol they all cause the same amount of growth, provided all other conditions are strictly comparable. That means that the influence of auxin on growth can only be explained on the basis of a chemical master reaction leading to growth, in which the molecules of the auxin take a part, and not by a stimulation “releasing” the “growth energy.”

The other part of the comparison between auxin and stimulus may seem more to the point. Auxin does not cause only growth in length, but it also affects other cell activities. If *only* auxin were involved in these cell activities, then auxin might be considered to set free one reaction or the other, that is to say, to stimulate, the cells determining what they were going to do once

auxin were there. But even in the writer's first paper on the growth hormone (WENT, 23) it was pointed out that in order to explain the auxin effect on growth it was necessary to assume a second factor, the food factor ("Zellstreckungsmaterial"). The experimental evidence for the presence of such a food factor was decidedly insufficient, but the theoretical foundation was sound. Later (WENT, 25) better experimental evidence for the food factor has been collected, so that a theoretical discussion of the relation of auxin to growth should include consideration of such a food factor. Recently LAIBACH (1936) arrived at similar conclusions; his second factor is identical with either the food factor or the auxin precursor.

Independently of any previous theoretical considerations it has been shown in this paper that it is not the presence of auxin which determines whether elongation, swelling, or root formation will take place in a pea stem, but rather more specific, independent factors, which have been named "calines." Their existence so far has not been directly proven, but enough evidence has been collected to make their existence highly probable. In addition the assumption of the presence and action of such calines offers the simplest explanation of many other effects related to growth and development of plants. A plant with cotyledons cut, that is, low in caulocaline but high in rhizocaline, will not grow in length or form swellings when auxin is applied, but will form roots in abundance; conversely, it is possible to get plants low in rhizocaline but high in caulocaline. They will form swellings without roots when concentrated auxin paste is applied to their apical cut surface. Also phyllocaline and caulocaline can be independently affected in the pea stem, causing good stem elongation without leaf growth, the reverse, both, or none. The possibility for the demonstration of these factors lies, like that for auxin, in their hormonal nature. They are formed or stored in one part of the seedling and are effective at another place. They disappear during their action, so that a constant supply is needed. Fortunately the different calines are formed or stored at different places in the plant. Caulocaline is formed in the roots, and the cotyledons have only a small storage, which becomes depleted in about one week after the removal of the roots. The stem contains an amount hardly sufficient for one day's growth. Phyllocaline is stored in the cotyledons, is formed in leaves in light, and is not present in the stem to any extent. As soon as the cotyledons are cut off, leaf growth stops. Rhizocaline also comes from the cotyledons, but it is present in the stem in considerable quantities, which only disappear gradually (in the course of 4 to 6 days when the roots are left on the stem, but the cotyledons removed). In a diagram representing a pea seedling (fig. 6) the place of formation of the various hormones is indicated.

A connection of auxin to phyllocaline is not clear or perhaps even non-existent, but, to make caulocaline or rhizocaline effective, the presence of

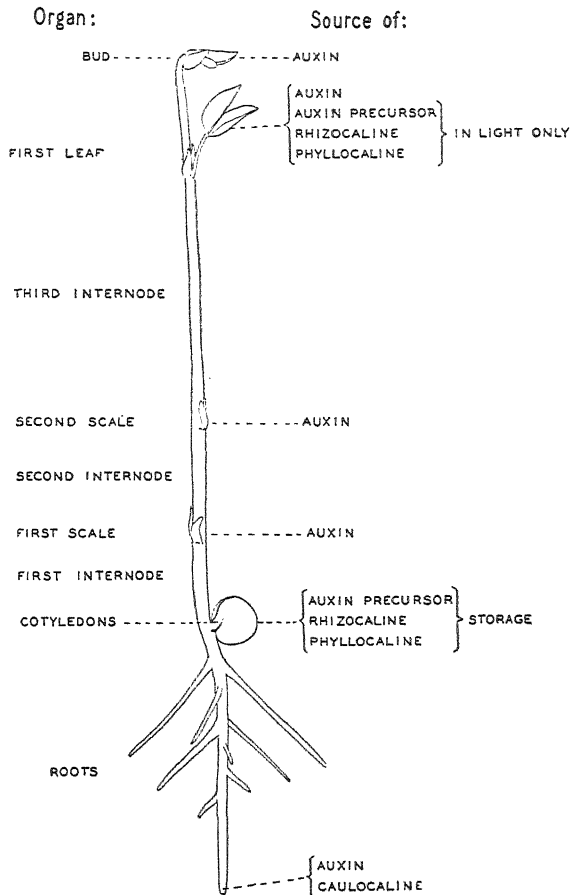


FIG. 6. Pea seedling with indication of places of formation and storage of various hormones discussed in the present paper.

auxin is required. The mutual relation between rhizocaline and auxin is the best investigated; hence it may be considered first. Without auxin no root formation is possible. If roots are formed without the specific addition of auxin it means that the plant contains some auxin already. But besides the necessity of the mere presence of auxin to render rhizocaline active, it affects rhizocaline in still another way. Auxin in suitable concentrations conditions almost quantitative accumulation of rhizocaline either near the base or near the apex of a cut stem. It is clear that it is not the auxin concentration in itself which is responsible for this accumulation, since no roots are formed in the middle portions of the stem, where, especially after treatment with high auxin concentrations, a high auxin content should be ex-

pected. It rather seems to be the auxin gradient which causes movement of rhizocaline. The naturally occurring polar auxin movement will cause an auxin accumulation near the base of a stem. If a very high auxin concentration is applied to the apex, then a second auxin gradient may be set up, especially when the auxin transport mechanism becomes inadequate to handle the large excess of auxin at the tip. How much rhizocaline will accumulate near apex or base, *i.e.*, the proportion of the total number of roots which will be formed at apex or base then depends upon relative magnitudes of the apical and basal gradients. Basal auxin treatment only will be effective when the concentration is high enough to cause basal uptake; it then will increase the normal gradient.

There is only one recent paper (COOPER, 2) which has a direct bearing on our subject. In it COOPER has shown that treatment of lemon cuttings with a high auxin concentration at the base results in an abundant root formation especially if the leaves are present. If, after the first auxin application, the treated portion of the stem is cut off and the cutting is treated again with auxin under the same conditions, no root formation beyond that of untreated cuttings results. This phenomenon COOPER explains on the same basis as used in this paper: the auxin causes an accumulation, near the base, of a second factor, rhizocaline, necessary for root formation. The rest of the cutting is then depleted in this factor, and retreatment does not have an effect.

Having seen how auxin affects the distribution of one of the calines in the plant, the same mechanism for the caulocaline may be examined. Lateral buds grow only when the apical bud is removed, and when enough caulocaline is available. Through the investigations of THIMANN and SKOOG (21, 22) and SKOOG and THIMANN (18) we know that the effect of the apical bud is exerted through its auxin. As long as it produces auxin the stem just below it grows, which means that it is supplied with caulocaline. If we now assume that auxin causes the caulocaline to move upwards in the stem and to accumulate near the place of auxin production, the dual effect of auxin on growth in length of stems, *viz.*, promotion of stem growth and inhibition of lateral bud growth no longer seem mysterious. As long as the caulocaline moves towards the apical bud, the lateral buds are not able to grow. But as soon as the apical bud stops forming auxin, the lateral buds with their slight auxin production are able to divert the caulocaline and grow. When the auxin production of the apical bud is supplanted by an artificial source, then also the caulocaline does not reach the lateral buds. Thus the inhibition of growth of the lateral buds is not a *direct* effect of auxin, but works through diversion of caulocaline. LAIBACH's view (13), that the lateral bud inhibition by auxin is due to its effect on the *growth* of other parts, has been disproven by SKOOG and THIMANN (18), but now the phenomenon can be ex-



plained through the effect of auxin on the *movement* of another growth factor. Whether accumulation of the caulocaline leads to growth or not is not important for the resultant bud inhibition.

The theory of direct inhibition through auxin can explain why an apical bud inhibits the basal buds, but it is at a loss to explain the opposite phenomenon. If, as mentioned in the section "stem and bud growth," a lower lateral bud or a cotyledonary bud overtakes the apical buds in development, then the latter are inhibited just the same. This cannot be caused by auxin, since in a plant the auxin formed moves downward only. But if the caulocaline is all used by the lower bud, the upper will be inhibited. The recent experiments of LE FANU (14) and SNOW (20) probably can find an explanation along the two-factor scheme as well.

Since the effect of roots on growth has been shown to occur through the intermediary of a hormone-like factor, which can move through the living plant, it would be very interesting to reconsider all cases in which an effect of roots on growth or development of other parts of the plant has been described. This would require a monographic treatment of the subject, which had better be postponed until more is known about caulocaline and possible other factors. A few cases might still be mentioned, if only to indicate how the facts discovered on etiolated pea stems have a much wider application and afford a better understanding of many plant growth processes.

The most marked effects of roots on stem growth have been found in the rootstock-scion relation. Such effects of one individual on another across a graft-union have generally been attributed to differences of root systems in regard to uptake of mineral nutrients from the soil (*e.g.*, WINKLER, 28). We have seen, however, that the root system can influence the growth of the stem through a more specific hormone-like agent, caulocaline. A root system, which forms only small amounts of caulocaline, will cause stunted growth in the scion, even when the root system itself seems adequate as far as its size is concerned.

Another clear case of the effect of the presence of roots is provided by greenwood cuttings. The growth of such cuttings stops completely after a short time. It is not resumed until roots have been formed. Growth is then proportional to the extent of rooting of the cutting. Thus it is very easy to tell by the growth of the cutting whether it has rooted or not.

One of the methods to keep plants stunted or dwarf-like is to cut their roots far back or to prevent their growth otherwise (see MOLISCH, 17), which of course will interfere with the caulocaline supply towards the stem and branches.

Remarkable and illuminating is also an analysis of the figures of HAGEMANN (8). He made leaf cuttings by sticking the petiole in the sand of the propagating frame and noted the time of first appearance of roots and

sprouts. He examined many hundreds of species, and found that only 32 per cent. of the species tested never formed any regenerates on their leaves or petioles; 42 per cent. formed roots only; 2 per cent. sprouts only; and 24 per cent. formed both roots and sprouts. From these figures it is at once clear that there is a close correlation between the formation of roots and sprouts; only when the former are present do sprouts also appear. But the correlation is still more pronounced. In the 142 plants which formed both sprouts and roots, not a single one had sprouts before the appearance of the roots; in 19 the sprouts appeared rather soon after or simultaneous with the roots, but in most cases it took 3 to 6 times longer for sprouts to form than for roots. This means that roots had to be present before sprouts would develop. The interpretation of these facts is evident. Leaves produce both auxin and rhizocaline. So roots can be formed on petioles, provided all other conditions are favorable. Once the roots are there, then caulocaline becomes available, and with the auxin from the leaves, sprouts are able to develop.

In the case of deciduous plants, which store not only storage food but also hormones, the necessity of leaves and roots for development will not be so apparent.

### Summary

1. It has been shown that, although auxin is necessary for growth in length of stems, apical swellings, and root formation in pea seedlings, it is not the only specific growth factor involved in these processes. It could be shown that also the presence of other hormone-like factors, "calines," is required. Without caulocaline, which is formed in roots, no elongation of the stem or lateral buds takes place. Probably the same factor is necessary for the development of swellings after treatment of the apical zone of the stem with a high auxin concentration. Rhizocaline, coming from the cotyledons, must be present to cause root formation in conjunction with auxin. Phyllocaline is necessary for leaf growth.

2. Auxin causes a redistribution of the calines in the plant, and with the aid of this phenomenon polar root formation and bud inhibition can be further explained. The specificity in development, the decision as to whether under the influence of auxin roots will develop, or growth in length or thickness will take place, depends on the relative concentration of the various calines. They belong to a new group of plant hormones. So far it has been impossible to handle them outside living tissue.

CALIFORNIA INSTITUTE OF TECHNOLOGY  
PASADENA, CALIFORNIA

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## BUD REGENERATION AND ELECTRICAL POLARITIES IN *PHASEOLUS MULTIFLORUS*

W. S. REHM

(WITH FIVE FIGURES)

### Introduction

Various investigators have reported experiments on the relationship between the growth substance and the inhibition of the lateral buds in seedlings of Leguminosae. THIMANN and SKOOG (24) and SKOOG and THIMANN (20) have demonstrated that the growth hormone when applied to the cut surface of decapitated plants of *Vicia faba* reversibly inhibits the lateral buds. HITCHCOCK (5) has shown that lateral bud inhibition could be produced by the application of various chemicals, including indole-acetic acid, indole-propionic acid, or ethylene or propylene gases to decapitated tobacco plants. THIMANN and SKOOG (24) have advanced the hypothesis that the growth substance is transported directly to the buds where it prevents the buds from producing their own hormone, which, according to these investigators, prevents the growth of the buds. LAIBACH (9), on the other hand, supposes that the hormone does not act directly on the buds but produces a secondary reaction which in turn causes the inhibition. In this connection it is of interest to note that LEFANU (10) was unable to detect any appreciable amounts of auxin in inhibited lateral shoots of *Pisum sativum*. BOYSEN JENSEN (1) obtained no release of inhibition when he applied growth hormone in various ways to the inhibited buds of several plants. SNOW (22) has demonstrated that there is an inhibiting influence that may travel both apically and basipetally. However, in a recent paper SNOW (23) found that the growth substance could travel upwards and cause both growth and inhibitory effects. Most of the workers in this field have found that the growth substance in physiological concentration travels only downward. Obviously the mechanism by which the growth hormone may bring about the inhibition of the lateral buds is one that has not been completely explained.

The present paper is concerned with the relationship between bioelectric currents and bud inhibition. A number of investigators have presented evidence to show that the hormone may be transported electrophoretically by the inherent potential differences. WENT (25) has proposed, on the basis of his experiments on the uptake of dyes (which experiments have been confirmed by CLARK, 4), that the apices of these plants are negative to their bases and that this electrical polarity transports the hormone. A discussion of this theory will be postponed until later. It must be kept in mind, however,

that the longitudinal transport of the growth substance is not necessarily the only rôle that bioelectric potentials might play in these phenomena. On the basis of the present status of the work in this field, it is possible that bioelectric potentials may influence the distribution of the growth hormone in the nodes of plants with inhibited lateral buds, or they may be involved in this phenomena in some as yet undetermined manner.

In a previous paper (17) the writer reported that he found in intact vegetative plants of *Phaseolus multiflorus* electrical polarities of relatively constant orientation in the regions of the axillary buds. In that paper a method was described for measuring potential differences between various loci of the plant. The loci between which the potential differences were measured are labelled in figure 3A. The potential differences between  $A_1B_1$ ,  $A_1P_1$ , and  $A_1P_1'$  were designated as the first node potentials, similarly,  $A_2B_2$ ,  $A_2P_2$ , and  $A_2P_2'$  as the second node potentials. In "unstimulated" plants, A was found to be negative in the external circuit to the other loci at a given node and this orientation was referred to as the normal one.

A portion of the work reported here was done at the University of Texas. For the experiments done there, the same method was used as that described in the previous article. The plants were raised in a greenhouse and placed in a light chamber 24 hours before an experiment; the potentials were measured either with a potentiometer or with a Compton Quadrant Electrometer. During this last year the writer has been continuing this work as a guest of the Department of Botany of the University of Chicago. He wishes to take this opportunity to thank Professor CHARLES A. SHULL for the many courtesies extended to him. At Chicago the plants were grown and measured in a greenhouse, the potentials being measured with a Compton Quadrant Electrometer. Because of the variation of the temperature during the day, experiments involving short periods of time were performed at night in the greenhouse. Under these conditions the temperature did not usually vary more than 2° C. during any given experiment. It must be emphasized that the plants used in the experiment reported here were all definitely in the vegetative state. Essentially the same results were obtained under the conditions of the light chamber and under the conditions in the greenhouse.

### Experimentation

#### INTERNAL POLARITIES

The first problem that the present paper is concerned with is an attempt to investigate the internal electrical polarities of the node. LUND (11) found, in his experiments on the Douglas fir, an internal polarity that was oriented oppositely to the external one. The possibility that the hormone

may be transported by the electrical currents makes it particularly pertinent to investigate the nature of the internal polarities.

For the purpose of measuring internal electrical polarities, small glass "pipes" of about one centimeter length were inserted into the plant so that the end of the pipe was at the center of the stem or petiole. Figure 1 shows

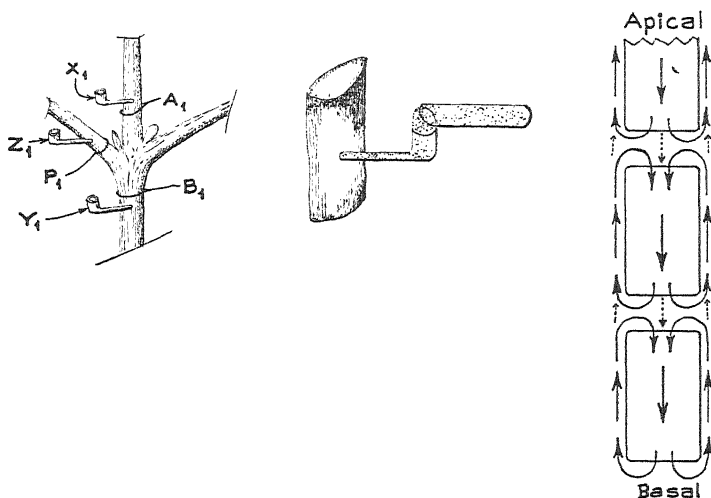


FIG. 1. Diagram on the left shows the position of the pipe contacts. The middle diagram shows the manner in which contact was made between the pipe and the contact cup. The diagram on the right represents a simplified portrayal of the possible path of current through the plant.

the method of leading off the potentials. The pipe was filled with Shive's solution and inserted into the plant. A contact cup of the same kind as shown in the previous paper (fig. 1) was filled with Shive's solution and brought close to the bowl of the pipe so that a drop of solution bridged the gap between the pipe and the contact without the contact actually touching the pipe. This arrangement permitted the plant to move without the stimulation that a rigid contact would produce. A  $\text{Zn}:\text{ZnSO}_4$  electrode was placed in the contact cup and the potentials were measured in the usual way. The first measurement after placing one of these contacts in the stem showed a decided negativity of the internal contact with respect to an external contact. That is, for example,  $A_1$  was always markedly positive in the external circuit to  $X_1$  immediately after the pipe contact  $X_1$  was placed in the stem. The potential difference between two such contacts decreased with time and in  $\frac{1}{2}$  hour to 2 hours reached a fairly constant value. After some preliminary experiments the following definitive experiment was performed. Four intact plants were taken and contacts were placed as shown in figure 1. Since the potential difference between an outside contact and an inside contact reached

a constant level within 2 hours, it was assumed that 4 hours would allow time for the immediate effects of injury to disappear. Consequently, 4 hours after placing the contacts on and in the plant, readings were taken between the various loci at fifteen-minute intervals over a period of 4 hours. These experiments were performed in the greenhouse at night. The temperature varied from 23° C. to 24° C. The results are shown in table I. It will be

TABLE I

ORIENTATION AND MAGNITUDE OF THE INTERNAL AND EXTERNAL ELECTRICAL POLARITIES IN THE FIRST NODE. LOCI OF THE VARIOUS CONTACTS INDICATED IN FIGURE 1.  
POTENTIAL DIFFERENCES IN MILLIVOLTS. FOR FURTHER  
EXPLANATION SEE TEXT

PLANT	$A_1B_1$	$A_1P_1$	$X_1Y_1$	$X_1Z_1$	$A_1X_1$	$B_1Y_1$	$P_1Z_1$
	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>
1 .....	-30	-15	-29	-29	+10	+11	-4
2 .....	-25	-25	-14	-14	+4	+15	+15
3 .....	-25	-20	-17	-19	-3	+5	-2
4 .....	-15	-10	-26	-15	-1	-12	-6

noted that the node potentials  $A_1B_1$  and  $A_1P_1$  possessed the normal orientation during this period. The potential differences  $X_1Y_1$  and  $X_1Z_1$  are all negative, that is,  $X_1$  is negative in the external circuit to  $Y_1$  and  $Z_1$ . This demonstrates that the internal polarities measured by this method possess the same orientation, *i.e.*, apical negativity, as do the polarities measured on the outside of the plant. The potential differences between an outside contact and the corresponding internal one are given in columns 5 and 8. The radial polarities, as can be seen from an examination of these measurements, are not as constant as the longitudinal polarities. On the basis of these results, a simplified diagram that would be consistent with both the internal and external longitudinal polarities having the same orientation is presented in figure 1.<sup>1</sup> This diagram represents the possible paths of current flow. It may be pointed out according to this diagram that the measured potential differences may be only a small fraction of the total RI drop in the plant. It would also follow that the total amount of current that can be drawn off from the plant may represent only a small fraction of the actual current density in certain regions within the plant. On the basis of the present experiments it is obviously impossible to say just where the cells producing the electrical potentials are located or whether or not there may be oppositely oriented gradients between the pith and the cortex. In this simple diagram no attempt has been made to represent the radial polarities.

<sup>1</sup> For evidence that the individual cells give rise to the measured potential differences in plants the reader is referred to the work of LUND (12), MARSH (14), and ROSENE (18).



## EFFECT OF DECAPITATION ON THE NODE POTENTIALS

In determining the effect of decapitation on the node potentials, it is necessary to eliminate the effect of mechanical stimulation at the contacts. A sharp movement of the stem or petiole against one contact caused the stimulated region to become more negative with respect to an unstimulated region. That is, if the plant was stimulated in the region of  $A_1$ , this contact became more negative with respect to  $B_1$ ; and if stimulated at  $B_1$ ,  $B_1$  became more negative to  $A_1$ . After stimulation, the potentials returned to normal in 5 to 15 minutes. To prevent this type of stimulation the plant was clamped between the node where the potentials were measured and the point of decapitation. A clamp with a large diameter was placed around the plant and the space between was filled with modelling clay; this procedure did not injure the plant in any observable way. The transmission of electrical variations produced by a mechanical stimulus has been reported for various plants by several investigators (BOSE, 2, HOUWINK, 6). However, in these experiments it was found that bending the plant above the clamp or giving it a sharp blow with a glass rod produced no observable effect on the node potentials. It must be pointed out that the period of the instruments employed in the experiment reported here was 15 seconds at the minimum and that electrical variations of shorter duration than this may have escaped detection. However, these experiments in which no effect was observed in the node potentials serve as an adequate control for the following experiments on the effect of decapitation.

Decapitation of the plant produced an immediate response in the potentials of the node below the point of decapitation. In the course of various experiments, more than 30 plants were decapitated below the second node and the potentials of the first node measured. At least 5 plants were used in each of the other experiments of this group. The plants were always clamped to prevent movement against the contacts. The plants were decapitated directly above the clamp with a sharp razor. The temperature at which the various experiments were carried out varied from 20° to 28° C., though the temperature did not usually change more than one degree during the course of any one experiment.

Figure 2 shows the typical effect of decapitation below the second node on the potentials of the first node. At the time indicated by the first arrow the plant was decapitated and the node potentials greatly increased in magnitude,  $A_1$  becoming more negative with respect to  $B_1$ ,  $P_1$ , and  $P_1'$ . The magnitude of the potentials then diminished, and in about an hour they had returned to their former magnitude. The next experiments were designed to determine if this response was caused by the loss of the apex or simply by the effects of cutting. A typical response to cutting of the stem of a decapitated plant is shown in figure 2A. At the time indicated by the second arrow

a section was cut from the top of the decapitated plant. This response was similar to the first, although of smaller magnitude and duration. Even though much longer periods of time (up to 24 hours) elapsed before the second cut, this response was always of smaller magnitude than that following the original decapitation.

It was mentioned above that certain mechanical stimuli (bending and hitting) failed to elicit a response when applied above the node. However, cutting or crushing the stem of an intact plant produced a response in the

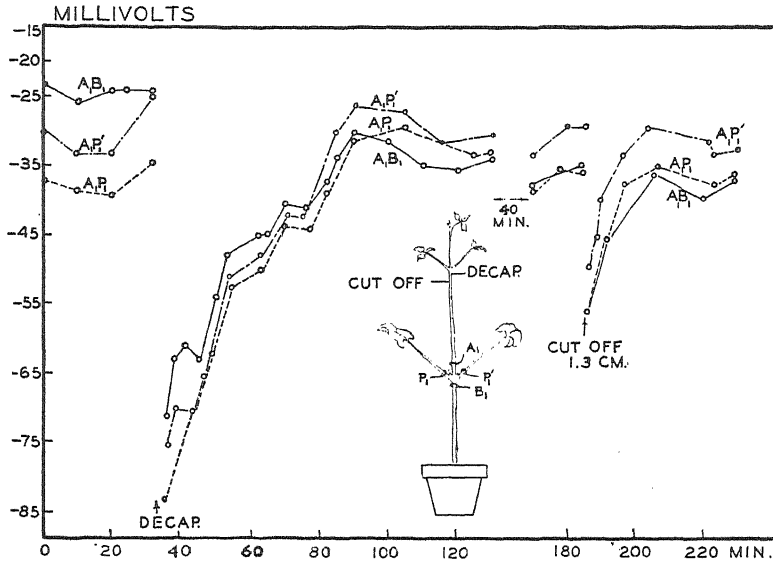


FIG. 2. The effect of decapitation on the node potentials of the first node. At the time indicated by the first arrow, the plant was decapitated. At the time indicated by the second arrow, 1.3 centimeters of the top of the decapitated plant was cut off.

node potentials. B, figure 3, shows a typical response to pressure on the stem. In this experiment the stem was squeezed with a pair of forceps with glass tubes placed over their tips. Gentle and fairly large pressures failed to produce a response in the node potentials. At the time indicated by the arrow, however, the stem was crushed by applying great pressure to the forceps. As can be seen from the figure, this caused an increase in the magnitude of the node potentials with a rather slow recovery. It was observed in plants decapitated between the second and third nodes that the buds of the first node often grew out as rapidly as those of the second node. Therefore the next step was to investigate the effect of decapitation on the first and second node potentials.

A, figure 3, shows the effect of decapitation below the third node on these potentials. The response of the second node potentials is seen to be similar

in magnitude and duration to the response of the first node potentials as represented in figure 2. In the first node potentials of the plant in A, figure 3, there was a small response in which the node potentials became slightly greater in magnitude. For convenience of presentation the response of the node potentials  $A_1P_1$  and  $A_1P_1'$  are omitted in this figure; the response of these potentials was similar to that of  $A_1B_1$ . A similar change was found in the second node when the plant was decapitated below the fourth node. These responses of the second node below the point of decapitation were always small and sometimes were within the limits of normal variation of the

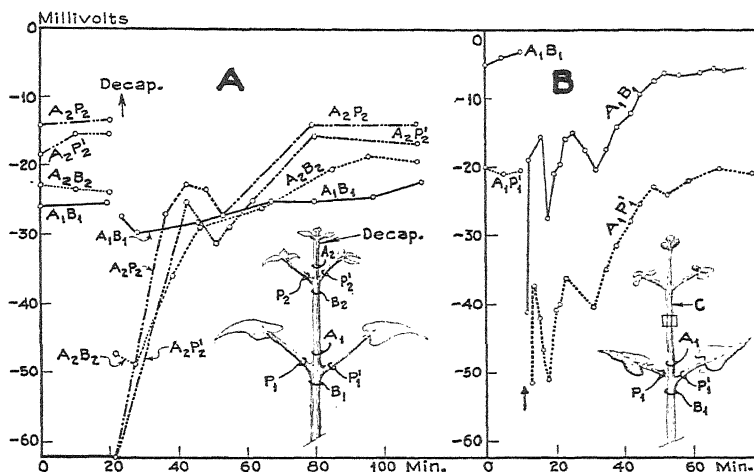


FIG. 3. A, the effect of decapitation below the third node on the node potentials of the first and second nodes. At the time indicated by the arrow, the plant was decapitated.  $A_1P_1$  and  $A_1P_1'$  are omitted from this figure for the sake of clarity. The response of  $A_1P_1$  and  $A_1P_1'$  was similar to that of  $A_1B_1$ .

B, shows the effect of crushing a portion of the stem on the first node potentials.  $A_1P_1$  is omitted; its response was similar to that of  $A_2B_1$  and  $A_2P_1'$ . The position of the clamp is shown in this figure.

node potentials. When the plant was decapitated below the fourth node, the potentials of the first node (*i.e.*, of the third node below the point of decapitation) did not change. At this juncture it may be pointed out that in 4 of these plants decapitated between the third and fourth nodes the buds of the first node grew out as rapidly as the buds of the higher nodes. In all experiments where the potentials of the node immediately below the point of decapitation were measured, a typical response, as shown in figure 2, and in A, figure 3, was observed. However, the particular shape of the recovery curves varied somewhat. The difference between the shapes of recovery curves in figure 2, and in A, figure 3, is not typical for the first and second nodes; the recovery curves of both nodes usually showed several plateaus or

dips. The experiment represented by A, figure 3, was selected in order to demonstrate that the recovery curves sometimes were suggestive of a diphasic variation. A discussion of the rôle of these responses in bud regeneration is postponed until later.

One important point to be brought out here is that, after the immediate response to decapitation, the node potentials return to approximately their former magnitude. The question then arises, is there any later response of the node potentials to decapitation, and if so, is there a relationship between it and regeneration?

#### REGENERATION OF BUDS AND THE NODE POTENTIALS

PROCEDURE.—In eleven plants the node potentials and the increase in length of the buds were measured for periods of from 80 to 120 hours after decapitation. Six of these plants were measured in the light chamber described in the preceding paper, and the other 5 were measured in the greenhouse. In 3 of the plants measured in the light chamber, the potentials were measured approximately every hour over a period of 100 hours. For the rest of the 11 plants the measurements were less frequent; usually readings were made at 1- or 2-hour intervals for two 6-hour periods each day. Ten of the plants, including the 3 plants measured at 1-hour intervals, were decapitated below the second node, and the first node potentials and the increase in length of the buds of the first node were measured. One plant was decapitated below the third node, and the buds and potentials of the first and second nodes were measured.

CHANGE IN NODE POTENTIALS.—For convenience of presentation the results are summarized as follows. The immediate effect of decapitation was typical in all plants. The node potentials showed, after the immediate effect of decapitation was over, spontaneous fluctuations similar to those described for intact plants in the previous paper. However, it was observed after averaging the readings of each successive 6- or 10-hour interval that the average magnitude of the node potentials in each case gradually diminished after decapitation. This diminution extended over a period of from 20 to 40 hours, and in the course of this time at least one and sometimes all of the node potentials became positive. The potentials continued to fluctuate at or above zero, though in some plants they definitely returned to normal before the end of the experiment.

Figures 4 and 5 show the typical course of the node potentials after decapitation. These curves represent 2 of the 3 plants referred to above in which readings were taken at approximately 1-hour intervals throughout the experiment. B, figure 4, represents the course of these potentials in one of the plants from the time of decapitation until about 40 hours afterward. Each point corresponds to a single reading. As can be seen in this graph,

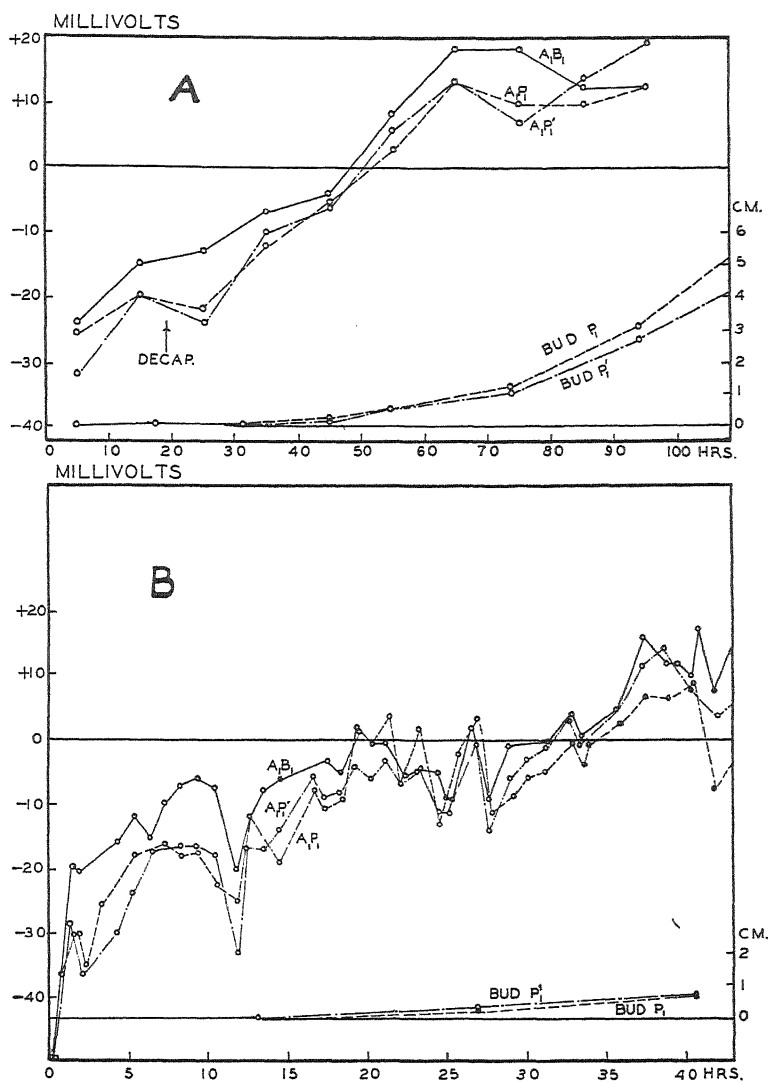


FIG. 4. Relation between the node potentials and the regeneration of the buds. A. Each point represents the average potential difference for ten hours. B. Each point represents a single reading.

these potentials fluctuate, and may return to a normal orientation for several hours, even after they have been inverted. For A, figure 4, and figure 5 the readings were averaged, each point representing a 10-hour interval. The change in the potentials shown in figure 5 is of average magnitude and illustrates the points brought out above. A, figure 4, representing the same

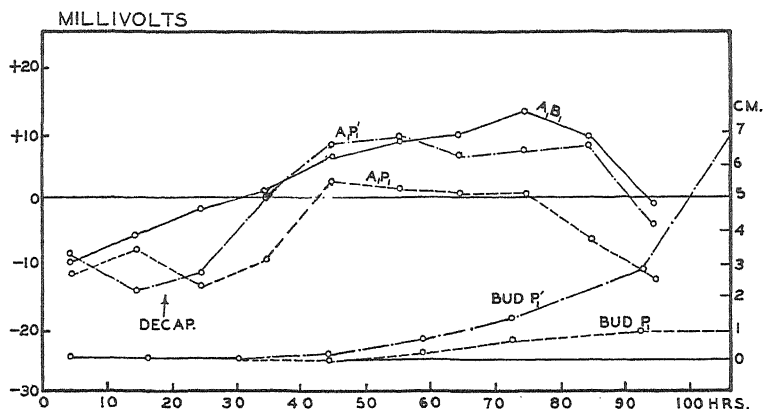


FIG. 5. Relation between node potentials and regeneration of the buds. Each point represents the average potential difference for ten hours.

plant as B, figure 4, also illustrates these points; but in this instance the change in the magnitude of the potentials was greater than usual.

TABLE II

COMPARISON OF THE AVERAGE OF THE NODE POTENTIALS IN DECAPITATED AND INTACT PLANTS. EACH NUMBER REPRESENTS THE AVERAGE OF THREE NODE POTENTIALS,  $A_1B_1$ ,  $A_1P_1$ , AND  $A_1P_1'$ , FOR AN INDIVIDUAL PLANT. POTENTIAL DIFFERENCES IN MILLIVOLTS. FOR FURTHER EXPLANATION SEE TEXT

PLANT	DECAPITATED PLANTS			INTACT PLANTS		
	20 HR. BEFORE DECAP.	1ST 30 HR. AFTER DECAP.	2ND 30 HR. AFTER DECAP.	1ST 20 HR.	NEXT 30 HR.	2ND 30 HR.
1 .....	mv. -23	mv. -9.6	mv. +10	mv. -27.5	mv. -25	mv. -26
2 .....	-11	-2	+8	-30	-24	-34
3 .....	-8	+3	+10	-30	-35	-15
4 .....	-30	-15	-14	-15	-16	-18
5 .....	-37	-26	-8	-17	-14	-16
6 .....	-18	-13	-6.6	-13	-14	-9
7 .....	-19	-3	+12	-12	-12	-17
8 .....	-26	-14	-5	-11	-22	-8
9 .....	-25	-1	+1	-10	-16	-20
10 .....	-15	-12	-2	-12	-11	-15
Avg. ....	-21.2	-9.3	-0.5	-17.7	-19.4	-17.8

In table II the 3 node potentials of all 10 decapitated plants were averaged for 3 periods: (a) before decapitation, (b) the first 30 hours after decapitation, (c) and the second 30 hours after decapitation. For controls, the node potentials of 10 intact plants (from previous paper, 17) were averaged for three periods: (a) the first 20 hours, (b) the next 30 hours, (c) and the succeeding 30 hours. In each of the decapitated plants the average

of the node potentials was more positive for the first 30 hours after decapitation than for the period before decapitation, and more positive for the second 30 hours than for the first 30 hours. On the other hand, in the intact controls the average of the node potentials was approximately the same for all three periods. As a final check on the effect of decapitation on the node potentials, the potentials of the first and second nodes of two plants raised and measured in the greenhouse were followed for 100 hours. One of these plants was decapitated below the third node, while the other one was left intact. In the intact plant, all of the node potentials remained normal throughout the entire experiment, while in the decapitated plant the potentials of both nodes exhibited the typical change after decapitation.

**NODE POTENTIALS AND BUD GROWTH.**—The length of the buds was measured with a pair of calipers which were adjustable by a fine screw. A mark was made with India ink on the node at the base of the bud, and the distance between the top of the mark and the tip of the bud was recorded as the length of the bud. The limit of accuracy of this method was about 0.25 millimeter.

In 4 of the 11 plants in which both bud growth and node potentials were measured, the buds did not start to grow out until 60 or more hours after decapitation. However, the node potentials in these plants showed the typical change after decapitation and were therefore diminished or inverted for 40 or more hours before any observable increase in the length of the buds. In 7 of these 11 plants the buds started to grow comparatively soon after decapitation. In 4 of these 7 plants both buds grew out; and in the remaining 3 plants only one of the buds definitely grew out. In every one of the 7 plants referred to above the node potentials definitely diminished before the buds started to grow. However, a given bud may start to grow before the corresponding node potential has become inverted. This is illustrated in B, figure 4; bud  $P_1'$  started to grow before the node potential  $A_1P_1'$  had become inverted, although this potential had diminished and was of comparatively small magnitude.

In 4 plants in which both buds grew out, the node potentials  $A_1P_1$  and  $A_1P_1'$  followed one another fairly closely from the time of decapitation to the end of the experiment. This is illustrated in A, figure 4. On the other hand, in the 3 plants in which only one bud definitely grew out, there was a marked difference between the node potentials  $A_1P_1$  and  $A_1P_1'$ . In each of these plants the node potential corresponding to the inhibited bud was definitely less positive than the node potential corresponding to the growing bud. In two of these plants the inhibited bud did not increase in length during the entire experiment. However, in the third plant, illustrated in figure 5, the "inhibited" bud started to grow, but became inhibited before the end of the experiment. In this plant the node potential  $A_1P_1$ , cor-

responding to bud  $P_1$ , returned to the normal orientation simultaneously with the inhibition of bud  $P_1$ . Later the node potential  $A_1P_1'$  also returned to the normal orientation. In fact, as can be seen from an examination of the results presented in table III, the node potentials frequently return to normal after the release of the buds.

TABLE III

COMPARISON OF THE NODE POTENTIALS AND GROWTH OF THE BUDS IN DECAPITATED PLANTS.

COLUMNS 5 AND 6 REPRESENT THE LENGTH OF THE BUDS IN CENTIMETERS.

COLUMNS 7 AND 8 REPRESENT THE INCREASE IN LENGTH OF THE

BUDS DURING THE FOUR-HOUR INTERVAL. COLUMN 9 GIVES

THE POTENTIAL DIFFERENCE FROM  $P_1$  TO  $P_1'$

PLANT	$A_1B_1$	$A_1P_1$	$A_1P_1'$	$P_1$	$P_1'$	$\Delta P_1$	$\Delta P_1'$	$P_1P_1'$
	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>cm.</i>	<i>cm.</i>	<i>cm.</i>	<i>cm.</i>	<i>mv.</i>
1 .....	- 9	- 9	+ 4	0.50	0.55	0.00	0.08	+ 13
2 .....	+ 5	- 10	+ 6	0.70	2.50	0.00	0.30	+ 16
3 .....	- 31	- 30	- 14	0.48	5.70	0.00	0.70	+ 16
4 .....	- 20	- 3	+ 14	0.75	9.50	0.00	1.10	+ 17
5 .....	+ 13.5	- 9	+ 5	8.30	16.80	0.05	1.70	+ 14
6 .....	+ 4	- 11.5	- 12.5	1.30	1.65	0.14	0.16	- 1
7 .....	+ 21	- 5	- 3	4.00	6.20	0.20	0.40	+ 2
8 .....	- 6	- 15.5	- 12.5	6.85	5.35	0.75	0.85	+ 3
9 .....	+ 7	+ 5	+ 5	8.60	9.70	0.74	0.88	0
10 .....	- 20	- 3	- 5	7.30	12.30	0.55	0.76	- 2

For further confirmation of the relation between the inhibited bud and the corresponding node potential, 10 decapitated plants were selected, 5 of which had one bud growing and the other bud inhibited, while the remaining 5 had both buds growing. The node potentials and the increase in length of the buds of each plant were measured for 4 hours. This work was done in the greenhouse, and because of the variation of temperature during the day, the experiments were performed at night. The temperature did not usually vary more than one or two degrees during any experiment. The temperature limits for this group of experiments were from 20° C. to 24° C. In this series of experiments the length of the buds was measured with a horizontal microscope, giving an accuracy of about 0.1 millimeter. The results of these experiments are shown in table III. The average values of each of the node potentials are given in the second, third, and fourth columns. Columns 5 and 6 give the initial length of the buds in centimeters, and columns 7 and 8 give the increase in length of the buds during the four-hour period, also in centimeters. It can be seen from columns 7 and 8 of the table that, in the first 5 plants, the bud  $P_1$  remained practically or completely inhibited throughout the experiment, while bud  $P_1'$  definitely increased in length. On the other hand, in the last 5 plants, both buds increased in length. In the first 5 plants,  $A_1P_1$ , corresponding to the inhibited bud, was



definitely more negative than  $A_1P_1'$ , corresponding to the growing bud. In contrast with this, in the last 5 plants the average node potentials were of approximately the same magnitude. To clarify these results the potential difference between  $P_1$  and  $P_1'$  was calculated from  $A_1P_1$  and  $A_1P_1'$  and is given in the last column of the table. That this procedure is permissible has been amply demonstrated by LUND and BUSH (13), RAMSHORN (16), and CLARK (4). It may be pointed out here that the writer has tested this principle in *Phaseolus* and has found that it always holds. It can be seen that  $P_1P_1'$  is definitely positive in all of the first 5 plants, while in the last 5 plants it is comparatively small. That is, in plants with both buds growing out, the node potentials  $A_1P_1$  and  $A_1P_1'$  were of approximately the same magnitude, and therefore  $P_1P_1'$  was of small magnitude. In plants with one bud inhibited and the other one growing, the node potential corresponding to the growing bud was definitely more positive than the node potential corresponding to the inhibited bud. For example, if bud  $P_1$  was inhibited and bud  $P_1'$  was growing,  $A_1P_1'$  was more positive than  $A_1P_1$  and therefore  $P_1P_1'$  was definitely positive. Since these last experiments showed a definite correspondence between the electrical polarity from petiole to petiole and the conditions of the buds, the question arose as to whether or not there was also a correspondence between the electrical polarity of the buds themselves and the state of the buds.

In order to answer this question, 8 plants were chosen, 4 of which possessed buds of approximately equal size, while in the other 4 one bud was definitely larger than the opposite bud. In these experiments contacts were placed on the buds 1.5 centimeters from the center of the corresponding node. Measurements were taken between  $A_1$  and these contacts. In table IV,  $A_1C_1$  refers to the potential difference between  $A_1$  and the contact on bud  $P_1$ ; similarly  $A_1C_1'$  refers to the potential difference between  $A_1$  and the contact on bud  $P_1'$ . Measurements were taken at night in the greenhouse every 15 minutes over a 4-hour period and each value in the table represents the average for this period. The temperature limits for this series of experiments were from 18.5° C. to 21° C.

It can be seen in this table that  $A_1$  is definitely positive in every case to the contact on the bud. In each one of the first 4 plants in which a large difference in the size of the buds occurs, the potential difference represented by  $A_1C_1$  ( $C_1$  representing the smaller bud) was less positive than the other potential difference represented by  $A_1C_1'$ . This relationship is similar to the one found between the size of the buds and the node potentials  $A_1P_1$  and  $A_1P_1'$ . The average potential difference between  $C_1$  and  $C_1'$  was calculated from  $A_1C_1$  and  $A_1C_1'$  and is given in the last column. This potential difference is definitely positive in the first 4 plants, i.e., the base of the smaller and more inhibited bud is positive to the base of the larger bud. In the last 4

TABLE IV

COMPARISON OF THE POTENTIAL DIFFERENCES BETWEEN  $A_1C_1$ ,  $A_1C_1'$  AND THE SIZE OF THE CORRESPONDING BUDS,  $P_1$  AND  $P_1'$ .  $C_1$  AND  $C_1'$  WERE CONTACTS PLACED ON THE BUDS 1.5 CM. FROM THE CENTER OF THE NODE. THE LAST COLUMN REPRESENTS THE POTENTIAL DIFFERENCE FROM THE BASE OF THE SMALLER BUD TO THE BASE OF THE LARGER BUD

PLANT	$A_1C_1$	$A_1C_1'$	$P_1$	$P_1'$	$C_1C_1'$
	<i>mv.</i>	<i>mv.</i>	<i>cm.</i>	<i>cm.</i>	<i>mv.</i>
1 .....	+10	+34	3.1	12.2	+24
2 .....	+13	+24	8.3	16.8	+11
3 .....	+28	+37	8.6	15.0	+9
4 .....	+19	+32	5.4	11.0	+13
5 .....	+45	+46	5.6	8.5	+1
6 .....	+42	+48	8.6	9.7	+6
7 .....	+38	+36	4.3	4.5	-2
8 .....	+40	+32	4.7	5.2	-8

plants, the actual magnitudes of the potential differences of  $A_1C_1$  and  $A_1C_1'$  are greater than in the first 4 plants. On the other hand, the difference in magnitude between  $A_1C_1$  and  $A_1C_1'$  is definitely less; that is,  $C_1C_1'$  is of smaller magnitude in the last 4 plants. This relationship again parallels that found between the node potentials  $A_1P_1$  and  $A_1P_1'$  and the size of the buds.

The question then arose, is there a definite electrical polarity between the apical region of a large growing bud and a homologous region of the opposite smaller bud? Four plants were chosen that possessed buds of unequal size; in each case the large bud was over twice the size of the smaller bud. Contacts were placed on the stem directly below the apex of each and the potential differences were measured between these two loci at 15-minute intervals for a 4-hour period. These experiments were also performed in the greenhouse at night. The temperature limits were from 19.5° C. to 21° C. The apical region of the larger bud was found to be strongly negative to that of the smaller bud. The average potential differences for the four plants were -32, -37, -42, and -51 millivolts respectively. It is well known that the inhibitory influence travels from the growing bud to the inhibited bud. Since the inhibitory influence travels downward in intact plants, the question then arose, is there a similar polarity between the apex and the base of intact plants?

Eight intact plants were selected with 2 nodes developed and with an average height of about 1½ feet. In each plant one contact was placed on the stem directly below the apex and another contact on  $B_1$ , directly below the first node, and readings were taken every half-hour over a 4-hour period. The experiments were performed at night in the greenhouse; the temperature limits were from 21° C. to 25° C. The averages of these readings are pre-

sented in table V. In all of the plants except the second, in this table, the apical contact was definitely negative to the basal one.

TABLE V

POTENTIAL DIFFERENCE IN MILLIVOLTS BETWEEN THE APEX AND BASE OF  
EIGHT INTACT PLANTS

PLANT	1	2	3	4	5	6	7	8
	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>
P.D. ....	-51	+4	-70	-41	-48	-14	-32	-28

The question then arose: Is there a continuous potential gradient along the stem or are there polarities that are oppositely oriented to the main one? For an answer to this question the polarity of the second internode, *i.e.*, B<sub>2</sub>A<sub>1</sub>, was calculated from the data presented in table IV of the previous paper (17) and is presented in table VI of the present paper. It will be noted that the apical region of the second internode is positive to its base in all but one of the plants, although it was of small magnitude in plant number six.

TABLE VI

AVERAGE POLARITY OF THE SECOND INTERNODE, CALCULATED FROM DATA PRESENTED  
IN TABLE IV OF PREVIOUS PAPER (17)

PLANT	1	2	3	4	5	6	7
	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>	<i>mv.</i>
B <sub>2</sub> A <sub>1</sub> .....	+22.1	+19.5	-0.7	+21.9	+18.3	+1.7	+20.4

#### EFFECT OF APPLIED CURRENT ON THE INHIBITION OF THE BUDS

It is well known that the inhibitory influence can pass through a dead region in the plant (SNOW, 21). This was also found to be true in the plants used in this investigation. Therefore, since the potential differences are dependent on the living cells (17), the electrical current is not necessary for the transport of the inhibition, at least over short lengths of the plant. Nevertheless, the possibility remains that the bioelectric potentials may influence the transport of this inhibition in the living tissue. This is in a sense similar to the passage of the hormone in the oat coleoptile. The hormone can pass from the decapitated tip through agar to the body of the coleoptile. The hormone apparently passes through the agar by diffusion but the transport of the hormone in the coleoptile itself is too rapid to be accounted for in terms of ordinary diffusion (VAN DER WEIJ, 26).

Current from an external source was applied to intact plants in various ways, in order to find out whether or not an applied current could release the inhibition of the buds. The results were negative as far as the release of the buds is concerned, and are briefly summarized in the following paragraphs.

The strength of the currents varied from 1 to 50 microamperes and were sent through the plant with contacts similar to the ones used for measuring the potential differences. They were washed at regular intervals to prevent the products of electrolysis from coming in contact with the plant. The currents were sent in the following directions:  $A_1$  to  $B_1$ ,  $B_1$  to  $A_1$ ,  $A_1$  to  $P_1$ ,  $P_1$  to  $A_1$ ,  $P_1$  to  $P_1'$ ,  $B_2$  to  $A_1$ , and  $A_1$  to  $B_2$ . The buds never grew out unless the stem between the apex and the node was killed and had dried out, and then only after the buds had grown out in control plants decapitated at the same time the current was first applied to the intact plant.<sup>2</sup> If only a short portion of the stem was killed, the buds remained inhibited. Sometimes the injury extended to the buds themselves and killed them (failure to regenerate after decapitation). Currents up to 5 microamperes produced little injury up to the time that the buds of the decapitated control plants started to grow out.

Although the applied current did not release the inhibition of the buds, several other effects of interest were observed. Injury always occurred first at the locus at which the current entered, even when these loci through which the current was sent were continually washed. This observation is similar to that of SCHECHTER (19) on the polarity of the lethal action of electric currents on *Conocephalus*. When current was sent from  $P_1$  to  $P_1'$ , epinasty occurred in the petiole toward the positive pole, and hyponasty in the one toward the negative pole. This effect was most pronounced in young intact plants, definite effects being produced within 6 hours by a current of 50 microamperes and within 20 hours by a current of 5 microamperes. It was also observed that when current was sent from petiole to petiole of the first node of young plants with a growing second internode, bending occurred in the second internode (a region through which presumably no current was passing). The internode would first bend with the convex side towards the positive contact, i.e., the apex itself would move toward the petiole on which the negative contact was placed. The bend would later become reversed, the convex side moving toward the negative contact, and eventually (while the current was still flowing) the stem would become straight again. With a current of 50 microamperes, the stem would show a definite bend within 10 minutes after application of the current, and the whole process would last for about 2 hours. Currents of 5 microamperes would produce a definite bend within 30 minutes.

<sup>2</sup> Injury appears sooner at the loci where the current enters and leaves the plant than in the stem or petiole in between the contacts. Therefore, except in preliminary experiments, current was not actually sent at these loci but at a region 4 centimeters from the node, i.e., 3 centimeters from the original contacts; and when the first signs of injury appeared the contacts were shifted slightly.

### Discussion

The experiments presented in this paper indicate that there is a positive correlation between the electrical polarities and the inhibition and regeneration of the buds. However, many more experiments need to be performed before anything approaching a complete picture of the patterns of bioelectrical potentials in this plant can be presented. Nevertheless several definite relationships have been brought to light by these experiments. The fact that decapitating the plant produced an almost immediate effect on the node potentials which lasted for about an hour demonstrates that events happening at one locus of the plant can definitely modify the electrical processes at a distant locus. The hypothesis presented itself that this particular variation in the node potentials may in some way be a factor in the release of the inhibited buds. Aside from other considerations, it is evident from these experiments that this increase in the magnitude of the node potentials is not a necessary prerequisite for the regeneration of the buds of that particular node, since in many plants decapitated above the third node the buds of the first node regenerated as rapidly as those of the higher nodes, whereas the effect of decapitation of the node potentials was small at the second node below decapitation, and entirely absent in the third node below. Also, crushing the stem produced a response somewhat similar to, though of not as great magnitude, the response to decapitation; and the buds remained inhibited for long periods afterward. From these considerations one can at least tentatively conclude that this immediate response of the node potentials to decapitation is not directly related to bud regeneration.

The experiments on the later effects of decapitation indicate that there is a definite relationship between regeneration and the orientation and magnitude of the potential differences. One of the interesting observations on the later effects of decapitation on the node potentials is that they diminish almost to zero, or become inverted before visible elongation of the buds occurs.

The results on the orientation of potential differences in this plant tend to confirm WENT's hypothesis that the apices of this group of plants are negative to their bases.<sup>3</sup> He has also postulated that the negative radical of the hormone is transported electrophoretically towards the positive pole. The fact that the orientation of the potential difference in the second internode is opposite to the main polarity of this plant indicates on first inspection that the hormone could not be transported in the manner postulated by WENT in this internode. However, if one assumes that these potential differences existing in the plant give rise to currents and that a current flows downward, say in the region of the nodes, then there must obviously be a return current

<sup>3</sup> RAMSHORN (16) has reported what amounts to an apical positivity for several plants. In this connection it may be pointed out that both WILKS (27) and CLARK (4), working on the oat coleoptile, have reported an apical negativity in this plant.

flowing in the opposite direction. The experiments on the internal distribution of polarities suggest that the return circuit for current flowing upward in the region of the node is through the cells themselves. Obviously it becomes difficult to decide, from potential difference measurements alone, in what direction the hormone could be transported by these currents unless the exact path through which the hormone is transported and through which the current flows is known.

The observation that applied currents did not release the inhibition of the buds indicates that the bioelectric currents may not play a rôle in the longitudinal transport of this inhibition. In this connection it may be pointed out that CLARK (4) has presented evidence to show that the longitudinal transport of the hormone in the *Avena* coleoptile is probably independent of the measured bioelectric potentials. However, CLARK points out that there may be some subtle relationship between the bioelectric potentials and the transport of the hormone that escaped detection by his experimental methods.

The writer feels that certain considerations must be kept in mind when interpreting the results of applied current on living organisms. If we assume that the potential differences give rise to currents, then, as has been pointed out above, there must be a return flow of current. It is obvious that it would be impossible to duplicate exactly a particular pattern of currents in a complex tissue by the application of a current from an external source. An E.M.F. from an external source when applied to the plant will tend to send current in the same direction throughout that portion of the plant to which it is applied. Therefore it is possible to duplicate at best only one direction of current flow while at the same time the applied current is of necessity opposing the return flow of the inherent current. Thus while the duplication of one portion of the electrical pattern may accomplish some result that that portion of the inherent current may itself accomplish in the normal plant, this applied current is also producing an abnormal physiological state in adjoining regions. It is therefore interesting that even currents of small magnitudes do produce visible injury when applied to the plant.

The fact that certain morphological changes similar to those produced by the hormone are produced by the application of small currents, *i.e.*, epinasty, hyponasty, and bending, indicates that the bioelectric currents may in some cases alter the distribution or effect of the hormone in this plant, or in some other way affect the growth processes. The work of BRUNNER and AMLONG (3), KOCH (7), RAMSHORN (16), WILKS (27), and others indicates that there is some causative relationship between the bioelectric potentials and the growth process in the phenomena of phototropism and geotropism. However, not all of the results of these workers are consistent with the hypothesis that bioelectric potentials transport the hormone to the

measured positive pole. CLARK (4) has demonstrated that the hormone is transported in agar blocks only under comparatively large potential gradients.

### Summary

1. The internal electrical polarities in the region of the nodes were found to possess the same orientation as the external ones.

2. Decapitation of a plant caused a large increase in the magnitude of the node potentials in the node directly below the point of decapitation, a small effect on the 2nd node below, and no effect on the third node below. Cutting a portion from the stem of a decapitated plant produced a similar response, but of smaller magnitude. Crushing the stem of an intact plant also produced a somewhat similar response to decapitation, although of smaller magnitude.

3. After decapitation, the node potentials returned to their former magnitude in about one hour and then steadily decreased until they were either inverted or of small magnitude. This decrease in the node potentials always preceded the first signs of regeneration of the buds.

4. In decapitated plants with both buds growing, the potential difference between the base of the petiole on the side of the growing bud was found to be negative to the base of the petiole corresponding to the inhibited bud; while in plants with both buds growing, the potential difference between the loci were found to be of small magnitude. Similarly, in decapitated plants the base of the growing bud was found to be negative to the base of the inhibited bud; and in plants with both buds growing, this potential difference was of relatively small magnitude. It was also found in decapitated plants that the top of the growing bud was negative to the top of the inhibited bud.

5. In intact plants, the apex was found to be negative to the base.

6. In intact plants, the apex of the second internode was found to be positive to the base of this internode.

7. Currents applied in various ways across the nodes of intact plants failed to release the inhibition of the buds except after a portion of the stem was killed and had dried out. Certain effects of the applied current not directly related to bud inhibition were observed.

8. In the discussion it is concluded that the response of the node potentials to the immediate effect of decapitation is probably not a causative factor in the regeneration of the buds. It is pointed out that there is a definite correlation between the inhibited and regenerating buds and the bioelectric potentials. The interpretation of the experiments on the effect of applied current is discussed.

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# UPWARD MOVEMENT OF INORGANIC SOLUTES AS AFFECTED BY A GIRDLE<sup>1</sup>

HARRY F. CLEMENTS AND CHARLES J. ENGARD  
(WITH NINE FIGURES)

## Introduction

Much evidence has been obtained during the last few years relative to the question of the path of the upward movement of the greater portion of the inorganic solutes in plants. There appears to be little doubt that the xylem is the tissue chiefly concerned with this function. The purpose of this work, in addition to contributing more evidence to this conclusion, is to determine by use of a number of plants some of the effects which a girdle imposes upon the rise of inorganic solutes, and the relation of xylem anatomy to the ease with which solutes pass a girdle, a relationship first suggested by CLEMENTS (2).

## Historical

The historical development of this subject has been outlined in a previous paper (2). Briefly, during the last fifteen years CURTIS (4, 5, 6) has developed the idea that the phloem tissue, and not the xylem, is chiefly concerned with the movement of inorganic ions upward.

CLEMENTS (2), in 1930, presented data definitely showing that there is a great upward movement of inorganic solutes past a girdle. Further, he indicated the error in CURTIS's experiments as well as in the latter's interpretative logic, pointing out that his failure to have proper controls rendered his experiments irrelevant to the conclusion that the phloem is the path of inorganic solute translocation, and that he unwittingly demonstrated that the girdle affects the rise of salts in part through its effects on the xylem. CLEMENTS showed further that while the quantity of salts which passed the girdle was very large there was a retarding effect caused by removing a strip of phloem and the consequent exposure of the current year's xylem. This retarding effect, at least in part, appears dependent in magnitude upon the distribution of tracheae in the seasonal development of xylem.

More recently, HOAGLAND and BROYER (8) demonstrated rather clearly that the xylem is concerned with the rise of the bromide ion. The rate of movement in the xylem appears to be maintained even after a girdle is made until some little while later, when it suffers a progressive retardation.

It is apparent from these investigations that since the salts travel with the transpiration stream in the xylem, they are subject to the same phe-

<sup>1</sup> Contribution no. 55 from the Botany Department of the State College of Washington.

nomena experienced by the transpiration stream. The results of the experiments set forth in this paper verify that relationship.

### Methods

Eight campus trees and shrubs, and two fruit trees, were used in these experiments. The trees and shrubs were poplar (*Populus tremuloides*), willow (*Salix* sp.), dogwood (*Cornus stolonifera*) growing in two different localities, mountain ash (*Sorbus americana*), Russian pea (*Caragana arborescens*), lilac (*Syringa vulgaris*), honeysuckle (*Lonicera* sp.); and the fruit trees, apple (*Pyrus malus*, variety unknown) and crabapple (*P. malus*). These latter trees were growing under unirrigated conditions.

Two- or three-year-old stems were cut off about 1 cm. above a lateral bud and a ring of bark, including phloem and cambium, removed about 4 cm. below the bud or buds. The ring itself was about 0.5 cm. wide. The exposed xylem was protected as much as possible by grafter's wax. Controls taken at the time of girdling (beginning controls) consisted of similar stems removed at a point corresponding with the position of the girdle on the girdled stem. Another set of controls (end controls) was left on the plants to be removed when the girdled stems were collected.

The girdling was done and the beginning controls taken on April 18, 1936, just as the buds were beginning to develop. The beginning controls, six to fifteen for each plant, were taken to the laboratory and prepared for analysis. The girdled stems and their end controls were collected when they had passed their maximum rate of growth. The number of stems or "cuttings" was noted for each plant.

In all cases, the samples were dried in an oven at 80° C. and then ground to 40-mesh fineness in a power mill after weighing to determine total dry weight. Total ash content was determined upon duplicate samples blasted to constant weight in a muffle furnace. The ash contents are reported as percentages of the dry weight and as milligrams per cutting. Total nitrogen was determined by the usual Kjeldahl method, with no modification for nitrates and nitrites, and reported as percentages of the dry weight and as milligrams per cutting.

The anatomical work was done in the following manner: 1-cm. pieces of 2- or 3-year-old stems of each plant were cut and put immediately into turttox. Later, hand sections were made, stained in safranin, dehydrated in alcohol, and mounted in balsam by the usual method. Projection drawings were made from these sections.

### Results

A discussion of work of this kind cannot be complete without some mention of the ecology of the region in which the plants were grown. The region has a bunch-grass climax. Only 14.68 inches of rain fell during the year in

which these studies were made, although the average approximates 21 inches per year, most of which falls in winter and spring; hence the normal growing season, especially June, July, and August, is dry. The atmosphere becomes extremely dry (as low as 5–10 per cent.) and the light intensity at this altitude often attains a magnitude of 12,000 foot candles; hence the transpiration rate is excessive. The trees on the campus have been introduced, and will maintain growth only when water is supplied. The trees used in this experiment, growing in a region which will not naturally support even the larger shrubs, are often handicapped by the inability of the root systems to provide water rapidly enough to prevent wilting. The occasional lack of water, a consequence either of erratic sprinkling or excessive demand, is manifested in the xylem of many of the trees by the formation of false rings (see *Populus*, (fig. 8), and *Pyrus* (fig. 3). Plants growing under such adverse conditions maintain a precarious balance with the environment, and any further inhibiting factor introduced into such a plant will be amplified under these conditions. Speaking teleologically, a tree in this region has an intense struggle for existence and reacts very strongly to girdling.

The girdled stems and controls of *Lonicera* were cut and brought into the laboratory on May 27, by which date they had made excellent growth. Both opposite lateral buds had developed on each stem, and the resulting growth attained a length of approximately 30 cm. The apple and crabapple stems were taken on June 28, when they apparently had made their maximum growth. Growth in both plants, as well as that of the girdled and ungirdled stems of each, was very good. On July 8, the remaining cuttings were collected. Of these, dogwood A<sup>2</sup> made the best growth, the girdled stems nearly equaling the ungirdled stems in appearance and size. The girdled stem of dogwood M made about half the growth of the ungirdled stems, and were visibly affected by the girdle. Poplar, willow, and lilac made only mediocre growth, and the girdled stems of Russian pea made very little growth. Only one girdled stem of mountain ash grew, and this but slightly. Of all the plants studied this one showed the severest reaction to the girdle. The analytical data are tabulated in tables I and II.<sup>3</sup>

Inspection of these data permits two fundamental conclusions: (1) the girdle does not *stop* the upward movement of inorganic salts, but, on the contrary permits the passing of large quantities of mineral salts in the xylem of the plant; and (2) treatment being the same in all plants, the response to the girdle seems to be characteristic of (a) the particular plant and (b) the particular site.

<sup>2</sup> Dogwood in two localities was used. These are designated "A" and "M" to indicate that from each locality.

<sup>3</sup> A "shoot" or "cutting" (tables I, II) is taken to be one girdled stem cut off at the girdle, or one control stem cut off at a point corresponding to the point of a girdle on the girdled stems.

TABLE I  
TOTAL ASH CONTENT

GENUS	NUMBER OF SHOOTS	DRY WEIGHT	TOTAL ASH AS	ASH PER
		PER SHOOT	PERCENTAGE OF	SHOOT
		mg.	%	mg.
<i>Salix</i>				
1st control .....	15	633	2.30	14.56*
Girdled .....	10	920	5.34	49.13
2nd control .....	6	5160	7.03	362.75
<i>Lonicera</i>				
1st control .....	10	980	5.03	49.29
Girdled .....	8	3375	6.91	233.21
2nd control .....	7	4957	6.00	297.42
<i>Populus</i>				
1st control .....	15	831	4.46	36.26
Girdled .....	24	1254	5.15	64.58
2nd control .....	10	1900	7.35	139.65
<i>Syringa</i>				
1st control .....	15	1026	4.80	49.25
Girdled .....	12	4216	2.66	112.15
2nd control .....	12	9435	3.29	310.41
<i>Pyrus</i> (apple)				
1st control .....	16	2312	4.66	107.74
Girdled .....	11	5636	5.19	292.50
2nd control .....	8	4312	6.08	262.20
<i>Pyrus</i> (crabapple)				
1st control .....	22	1341	4.71	63.16
Girdled .....	16	4163	5.01	208.51
2nd control .....	12	6916	5.36	370.69
<i>Cornus</i> M				
1st control .....	13	679	2.07	14.06
Girdled .....	12	2475	4.40	108.90
2nd control .....	6	5833	7.49	436.89
<i>Cornus</i> A				
1st control .....	13	1115	2.09	23.30
Girdled .....	12	5708	5.22	297.90
2nd control .....	5	9080	6.77	614.72
<i>Caragana</i>				
1st control .....	15	1466	2.52	36.94
Girdled .....	12	2141	3.64	77.93
2nd control .....	8	2787	5.40	150.50

\* The amount of ash which passed the girdle in each case is the difference between the ash content of the girdled shoot and that of the first control, and, under equally ideal conditions for all species, seems to be correlated with the effect of the girdle on the xylem. The difference between the ash content of the girdled shoot and that of the end control is a measure of the effect of the girdle on the growth of the shoot, considering all factors influencing the rate of growth; but under ideal conditions would stand in an inverse relationship with the difference between the first control and the girdled shoot.

Considering now the first of these conclusions, it is readily evident from table I, that in all cases, the mineral materials continue to move into the shoots after the girdles were made. The largest increment of ash between the beginning control and the girdled stem is in dogwood A, which also exhibited the best foliar growth. The ash content of the girdled stems is about

TABLE II  
TOTAL NITROGEN CONTENT

GENUS	NUMBER OF SHOOTS	DRY WEIGHT PER SHOOT	TOTAL N AS PERCENTAGE OF DRY WEIGHT	TOTAL N PER SHOOT
		mg.	%	mg.
<i>Salix</i>				
1st control .....	15	633	0.854	5.41*
Girdled .....	10	920	0.816	7.51
2nd control .....	6	5160	1.080	55.73
<i>Lonicera</i>				
1st control .....	10	980	2.240	21.95
Girdled .....	8	3375	2.176	73.44
2nd control .....	7	4957	1.856	92.00
<i>Populus</i>				
1st control .....	15	831	0.744	6.18
Girdled .....	24	1254	0.552	6.92
2nd control .....	10	1900	0.992	18.85
<i>Syringa</i>				
1st control .....	15	1026	1.576	16.17
Girdled .....	12	4216	0.928	39.12
2nd control .....	12	9435	1.632	153.98
<i>Pyrus</i> (apple)				
1st control .....	16	2312	0.888	20.53
Girdled .....	11	5636	0.904	50.95
2nd control .....	8	4312	1.194	51.49
<i>Pyrus</i> (crabapple)				
1st control .....	22	1341	0.880	11.80
Girdled .....	16	4163	1.248	51.95
2nd control .....	12	6916	1.509	104.36
<i>Cornus</i> M				
1st control .....	13	679	7.228	4.84
Girdled .....	12	2475	1.016	25.15
2nd control .....	6	5833	1.301	75.88
<i>Cornus</i> A				
1st control .....	13	1115	1.024	11.42
Girdled .....	12	5708	1.104	63.02
2nd control .....	5	9080	1.827	165.89
<i>Caragana</i>				
1st control .....	15	1466	2.376	34.83
Girdled .....	12	2141	1.920	41.11
2nd control .....	8	2787	2.517	70.15

\* See footnote of table I.

13 times that of the beginning controls. Its nearest competitor on this basis, and in apparent growth, is dogwood M, which showed an ash content equal to approximately 8 times its check. *Lonicera* accumulated mineral materials in its girdled stems equaling 4.5 times that of the check; crabapple about 3.5 times the check; and the girdled apple stems a little less than 3 times their checks. The girdled stems of willow, and lilac approximately trebled their ash contents. The Russian pea and the poplar, the girdled stems of which had twice the ash content of the checks, had the smallest increment of ash of all plants used. *Sorbus* failed to grow. Thus there is a transport of inor-

ganic salts past the girdle, and obviously in the xylem, in quantities ranging from 13 down to 2 times the salt content of the beginning controls. It should be remembered that this salt movement was taking place in plants growing under difficult ecological circumstances.

Scrutiny of table II, which presents data on the total nitrogen content, reveals an increment gradient of the same pattern as that for the ash content. Here, as with the ash content, the two dogwoods yield the greatest increase in total nitrogen in mg. per cutting, the girdled stems of dogwood A showing a total nitrogen content of 5.8 times that of the beginning controls, and of dogwood M, 5 times the nitrogen content of the beginning controls. The girdled shoots of crabapple, *Lonicera*, and apple are next, with total nitrogen contents of 4.3, 3.5, and 2.5 times the content of the respective controls. Poplar and Russian pea are at the bottom of the list. The assumption here is, of course, that all nitrogen compounds present above the girdle were derived from nitrogen salts rising through the xylem, and synthesized in the leaf region above, a phenomenon known to be common among perennials (17). In *Populus* and *Caragana* the increase above the girdle is so small that it indicates a situation such as THOMAS (17) found, namely, that the inorganic nitrogen loses its identity in the roots and is changed to the organic, and as such may move through tissues other than xylem. This may, indeed, account for some of the poor growth made by some girdled shoots. It may be pointed out, however, that even the ungirdled end controls do not increase in nitrogen as much as they do in ash. Thus the total nitrogen per girdled shoot ranges from practically nothing in *Populus* to 5.8 times the nitrogen content of the beginning controls in *Cornus*.

These increases in the ash and total nitrogen content of the girdled stems are definite demonstrations of the thesis that large quantities of inorganic solutes rise in stems after the continuity of the phloem has been broken, and therefore lead to the conclusion that the xylem is the chief path of transport. That this conclusion is correct has been further verified by HOAGLAND and BROYER<sup>4</sup> who showed that xylem tissue carries the bromide ion, and, what is perhaps more significant, that the removal of a ring of bark did not greatly, if at all, affect the upward movement of this ion in the cotton plant for several hours, or in young citrus trees for much longer periods. This means, then, that a girdle, *per se*, in so far as it breaks the continuity of the phloem, does not affect the upward movement of salts, but that other factors, induced as a consequence of the girdling process, somehow interfere with the upward movement of the dissolved salts in the xylem. The dissolved salts are identified as being in the "transpiration stream," and would seem to be subject to the same forces.

<sup>4</sup> Private communication. The writers are also informed that some earlier work by Dr. F. C. STEWARD on movement of the bromide ion has been done, but the results have not yet been reported.



Turning now to the second conclusion derived from these studies, namely, that the effect of the girdle on the upward movement of salts appears to be characteristic of the species, one can arrive *a priori* at the conclusion that the distribution and size of tracheae and tracheids, and the cell wall thickness of water conducting tissues are important factors governing the ease, and therefore the rapidity with which the rising column of water with its dissolved salts can negotiate the girdle. It was this thesis which determined the course of experimentation pursued in this work.

A tracing of a projected cross-section of the xylem was made of each plant used in the investigation. These are shown in figures 1 to 9. They are correlated in table III with the data of tables I and II, and are found to

TABLE III

CORRELATION OF XYLEM ANATOMY WITH THE INFLUENCE OF A GIRDLE  
ON THE RISE OF ASH MATERIALS

XYLEM TYPE	GENUS	ASH PER SHOOT		
		1ST CONTROL	GIRDLED	INCREASE
		mg.	mg.	%
Diffused porous				
Shrub .....	<i>Cornus</i> A	23.30	279.90	1178.8
Shrub .....	<i>Cornus</i> M	14.06	108.90	674.5
Shrub .....	<i>Lonicera</i>	49.29	233.21	373.1
Tree .....	<i>Salix</i>	14.56	49.13	237.4
Tree .....	<i>Pyrus</i> (crab-apple)	63.16	208.57	230.2
Tree .....	<i>Pyrus</i>	107.74	292.50	171.5
Shrub .....	<i>Syringa</i>	49.25	112.15	127.9
Ring porous				
Shrub .....	<i>Caragana</i>	36.94	77.93	110.97
Tree .....	<i>Populus</i>	36.26	64.58	77.9
Tree .....	<i>Sorbus</i>	.....	.....	.....

correspond rather closely with the increment gradient of the ash and total nitrogen content. On the basis of the following observations, such a correlation might well be expected:

(1) The new ring of xylem is the chief functional region as regards sap rise, since it leads directly to the new regions of growth and activity, although some sap may rise in the xylem of the previous year, depending upon the quantity of gas held within the central cylinder (10).

(2) Because of an insufficient supply of elaborated foods from above, the production of new xylem below the girdle is considerably curtailed, while above the girdle the new xylem production is unimpaired because of an ample supply of carbohydrates moving downward from the leaves; in

fact, this growth above the girdle may even be stimulated under ideal conditions.

(3) The rising transpiration stream in the old xylem makes connection with the new xylem above the girdle by a lateral movement from the older vessels to the vessels of the new wood, especially where the distal portion of the experimental branch has been removed. Further, the girdling process, no matter how carefully done, causes injury to the xylem. Even though the exposed xylem is protected by paraffin or grafting wax, there is some incapacitation of it that is caused not by mechanical injury but by a plugging of the xylem elements with air drawn in by suction tension (10), or by a general disorganization caused by the isolation of the living cells of the xylem, which die as a result of girdling, as is evidenced by a discoloration of the exposed tissue. Also, the reduction in the cross-sectional area of the water channel itself, or at least the prevention of its development at the place of the girdle, will be effective in reducing water movement. It must be remembered that, if a girdle is made in the spring, the xylem which is exposed is that which was sufficient to provide water for the previous season's growth, but not for the greater foliage development of the present year. This fact must be taken into account whenever girdled and ungirdled stems are compared, because the actual conducting volumes in the two cases are unequal. To obtain a figure for the relative conducting volumes, girdled and ungirdled stems of *Cornus* were taken. The girdles were made July 15, at which time the plants were approaching their greatest size. Six weeks later, these shoots together with the ungirdled controls were removed. The stems were cut (a fresh cut made under water to prevent air clogging) and water was sucked through 10-cm. lengths to a suction flask. One of the 10-cm. lengths included the girdle, while the control was taken in juxtaposition to the girdled portion. An average of several 3-hour tests showed that the ungirdled stems had conducted 33.8 per cent. more water than the girdled stem. Since, unlike the field conditions, the force was distributed equally over the whole cross-sectional area, there was no lateral movement in the ungirdled stem; so the result is no more than the measure of the relative cross-sectional areas of the conducting tissue. In diffused porous woods in which the vessels lie next to the new xylem, there may be a lateral movement of the stream farther into the old wood because of the plugging of the columns next to the girdle. In a case of this kind there would be two lateral movements, one inward below the girdle and the other outward above the girdle.

(4) The rapidity and ease with which a lateral transfer from xylem of a previous year to that of more recent development is effected is apparently dependent upon (1) the proximity of the tracheae of the old xylem to those of the new xylem; (2) the size of the tracheae; (3) the number of tracheae and other elements through which the laterally moving solutions must pass; and (4) the thickness of the cell walls of all xylem cells.

A consideration of the mechanism of the lateral movement must be included before going farther. While the xylem rays may be instrumental in moving foods and salts from xylem to phloem and *vice versa*, it seems improbable that a mass movement of water would take place through them sufficient to supply the amount used by the transpiring leaves; while such a movement is possible, at best it would be extremely slow. The possibility that cell wall pits are operative in such a lateral movement, a postulate of STRASBURGER (16), has been discredited by MACDOUGAL, OVERTON, and SMITH (10). The fact that in the majority of plants the pits are found sometimes exclusively on the radial rather than the tangential walls, while lateral movement still takes place, would seem to further verify this criticism. It should be emphasized that the lateral movement of water, in this paper, refers to the movement of water from older to newer xylem, not merely a movement tangentially about the stem.

There remain, then, two possible means of lateral movement; a lateral movement through leaf or branch traces (10), and a lateral movement through the cellulose gels which constitute the cell walls. MACDOUGAL, OVERTON, and SMITH (10) showed that no dye was moved laterally to outer xylem layers from inner xylem layers to which dye had been added through a bore-hole. They concluded that there is no lateral movement, and could not demonstrate that movement took place through the traces. As the method by which the stream is transferred from the older layers of xylem into the newer layers, they suggested that the central column of xylem has a conical terminal which "is surrounded by a thimble-shaped mass of wood whose upper half (longitudinal growth of the present season) is solid, and whose lower half (the portion formed laterally to the previous year's wood) is hollow. It appears, therefore, that in the two-year-old twig, water entering the first year's wood moves more or less vertically through it to the region where the second year's wood caps that of the first year." Thus, the "lateral" movement as postulated by MACDOUGAL, OVERTON, and SMITH (10) is actually a vertical movement caused by relayed suction forces of the transpiring leaves of the current year. The results of their dye experiments seem to be consonant with such a mechanism, but in this work the stems are cut off above the lateral bud, making all such vertical transfers impossible. How, then, can the developing bud receive the influx of salts except by a lateral movement of the rising transpiration stream directly across the xylem, either through the cell walls or the lamellae? There appears to be no alternative.

In order to test this hypothesis some shoots of *Cornus* and *Caragana* were girdled on July 14. The tops of these shoots were not removed as in previous experiments. Six weeks later these shoots were removed at a point several inches below the girdle. These shoots were plunged into water, and a second

cut was made to prevent air plugging. They were placed in a solution of dye. After four days, they were removed and the course of the dye movement was determined. The dye had moved past the girdle in *Cornus*, but, while at the girdle only those vessels at a distance from the outside were colored, immediately above the girdle the dye was found in xylem cells next to the cambium. *Caragana*, however, showed no passage of the dye above the girdle, even though it apparently was receiving water. Thus, a lateral movement in *Cornus* is demonstrated, which in a sense contradicts the conclusion of MACDOUGAL, OVERTON, and SMITH. This further indicates that a girdle incapacitates the exposed xylem even though the latter is not structurally destroyed, since only those vessels at a distance from the exposed xylem cells conducted the dye.

In an earlier publication, MACDOUGAL (9) reports an experiment in which several small trees were girdled about September 1. A few days later a tree was cut, topped, and attached to a suction pump, with the base of the tree in fuchsin. The pump was operated at a vacuum of 74 cm. of mercury for 4 hours. At the end of the time 250 cc. of dye had been drawn through to a suction flask past the girdle. The dye had passed through in all layers of the xylem, with no variation of the conducting stream. Another of the girdled trees was cut a few weeks later and treated in the same manner. The time of pumping was from 3 P.M. one afternoon to 9 A.M. the next morning, and at the end of that time 200 cc. of dye had been drawn through at the normal rate of 8 cm. per hour. All layers of xylem were colored up to half the distance, and above that it was found only in the second and third layers of xylem, and "when the stem was bisected it was seen that the exposed wood formed during the current year had not conducted the dye, which had come up in this layer to the margin of the exposed zone, been blocked out of it across the girdle, and then *diffused into*<sup>5</sup> and followed it up many centimeters above, as has been noted."

In the system used in our experiments there is no equal tension on each xylem year because the stem is cut off. If the current development of xylem is inhibited below the girdle, the transpiration stream rising primarily in the previous year's xylem is drawn past the girdle laterally into the current xylem above the girdle by a "one sided" suction tension from the leaves. Obviously this situation is very different from that of the dye injection experiments of MACDOUGAL, OVERTON, and SMITH, and of MACDOUGAL's experiment.

This lateral movement, seemingly a "soaking process," operates upon the cell wall which is regarded as a cellulose gel. From recent x-ray work by SPONSLER (12, 13), SPONSLER and DORE (14), ASTBURY, MARWICK, and BERNAL (1), and others much light has been thrown upon the structure of

<sup>5</sup> Italics ours.

the cell wall. Cellulose is regarded by these workers as a double anhydride of glucose of the empirical formula  $C_6H_{10}O_5$ , four of which are bound together to form the unit. The dimensions of the unit are 8.35 Å, 10.34 Å and 7.0 Å. The micelle of cellulose consists of elongated groups of 40 to 60 long chains, each being 50 to 80 hexan units in length, the anhydrides being held together by strong primary valence forces. Wood, however, is more than just cellulose. The primary or fundamental cell-membrane or fiber is cellulose, which is converted into wood by lignification or an "incrusting" of the cellulose with "lignin," a collective term referring to a group of heterogeneous organic substances.

These substances of carbohydrate derivation exist in wet conditions as gels, whose swollen volume is determined by the amount of water contained. The micelles of cellulose evidently are much closer when a fiber is dried than when it is swollen, according to FREY (7). CRAFTS (3) has presented a diagram showing cell walls much swollen in the natural condition and shrunken when partly dried or fixed for histological purposes. Further, cellulose swells more in solutions of electrolytes than in pure water, the reason being that "one ion is absorbed more than another. This separation of electric charges results in the stronger binding of the electric dipole of water; it also charges all of the cellulose micelles in the same sense, thus causing them to repel one another. This repulsion separates the micellar structure and allows water to enter, as it were, passively into the increased interstices" (11). The transpiration stream is, among other things, a weak solution of electrolytes. The fact that swelling takes place indicates that water is taken into the structure; and when a considerable "suction" force is applied at one side of the gel, it seems reasonable that water can be drawn through the avenues which are present as micellar interstices.

The thicker the cell wall, the more difficult will be the passage of solution through it, since a greater total-resistance will be encountered. If the lateral movement takes place across a great many small, thick-walled tracheids, the movement would be much slower than across a few thin-walled tracheids. But even where this lateral movement is comparatively easy, it is more difficult than a vertical movement through the lumina of the vessels.

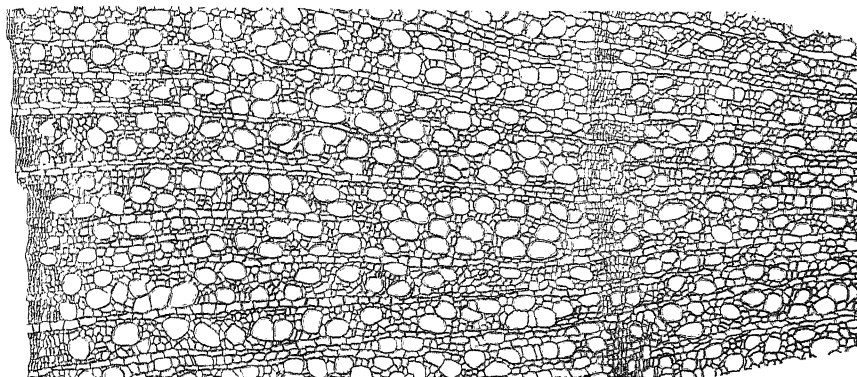
There are two general types of xylem as regards tracheal distribution: the "ring-porous" type, in which the tracheae are concentrated in the spring growth of xylem, forming a more-or-less definite ring; and the "diffuse-porous" type, in which the tracheae are scattered evenly throughout the whole annual ring. There are, of course, all degrees of intergradation between ring-porosity and diffuse-porosity, a "semi-diffuse" porous xylem representing a median where large and closely packed vessels are concentrated in the spring wood, with vessels becoming increasingly smaller and less frequent throughout the summer wood, with few or none in the late summer wood at the end of the year's growth.

The xylem of the plants used in this experiment represent a rather complete series of the above types, and, further, in one of them, *Sorbus*, the production of tracheae has been reduced to only scattered groups of six or eight cells per group, those generally in the spring and early summer wood. For convenience in correlating the xylem anatomy of these plants with their corresponding ash data the following grouping of the plants is used and the correlation is made with the analytical data in table III.

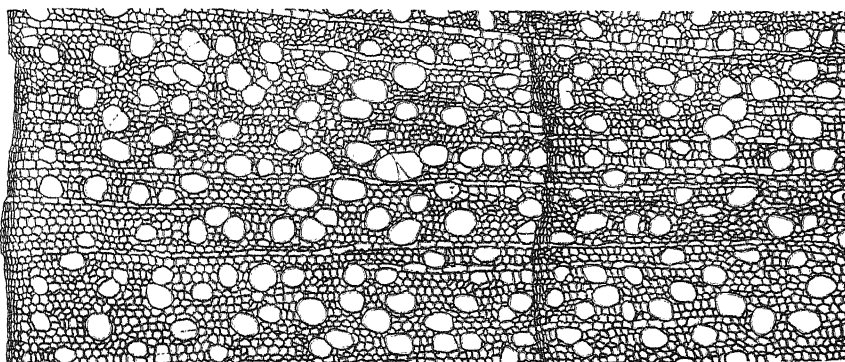
In this table, two groups are made: the plants with diffuse-porous xylem, represented by dogwood, apple, honeysuckle and willow; those with ring-porous xylem, represented by poplar, lilac, and Russian pea; and the mountain ash with its paucity of vessels but so arranged that it does not fit into either of the first two groups. On the basis of the ability of the xylem to carry the solutes when girdled, these plants arrange themselves in order of decreasing amounts as follows: dogwood A, dogwood M, honeysuckle, willow, crabapple, apple, lilac, Russian pea, poplar, and mountain ash. Figures 1 to 9 reveal that, on the basis of xylem structure with reference to tracheal number and distribution, the order from greatly diffused porous xylem to ring porosity is as follows: crabapple, dogwood A, dogwood M, apple, honeysuckle, willow, lilac, Russian pea, poplar, and mountain ash. Were it not for the fact that the two apple trees are out of line, the series would be exactly parallel. The behavior of the apple trees, however, is explainable when it is remembered that they were not watered, but were growing under difficult ecological conditions. This emphasizes the fact that whatever influences the transpiration stream also influences the movement of soil solutes, although not necessarily in proportion.

There is no sharp line of demarkation between diffuse-porous and semi-diffuse-porous, but for the sake of the grouping, crabapple, apple, dogwood, and *Lonicera* have been placed in the diffuse-porous group. If one inspects figures 1 to 4<sup>6</sup> one will have no doubt that crabapple and *Cornus* are diffuse-porous. In these two plants the vessels are numerous, large, and evenly dispersed. There are 6 or less tracheids separating the vessels of one annual ring from another in both of these plants. In crabapple, however, the vessels are much more numerous, and the tracheids separating them are fewer than in *Cornus*; for this reason crabapple has been placed first in the order, and *Cornus* second. In the apple, the vessels are less numerous than in crabapple, and much smaller than in crabapple and *Cornus*. Here, also, there is just a suggestion of departure from the strictly diffused porous type, manifested by a slightly greater concentration of tracheae in the spring wood. The vessels of the spring wood are also slightly larger than those of later summer. The tracheae of *Lonicera* (fig. 4) are scattered throughout

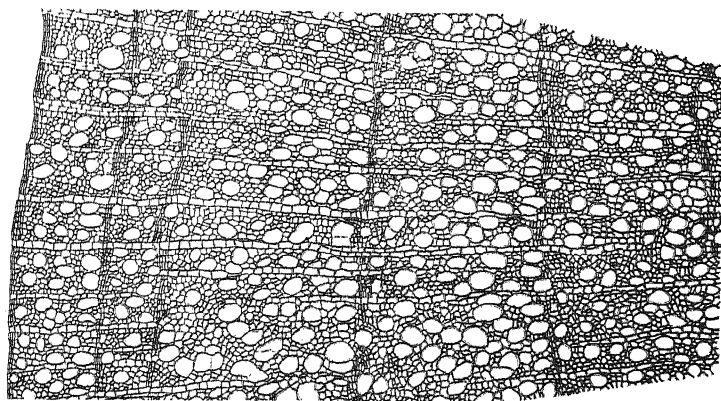
<sup>6</sup> The writers are indebted to DOROTHY ENGARD for her assistance in making the xylem drawings.



1



2



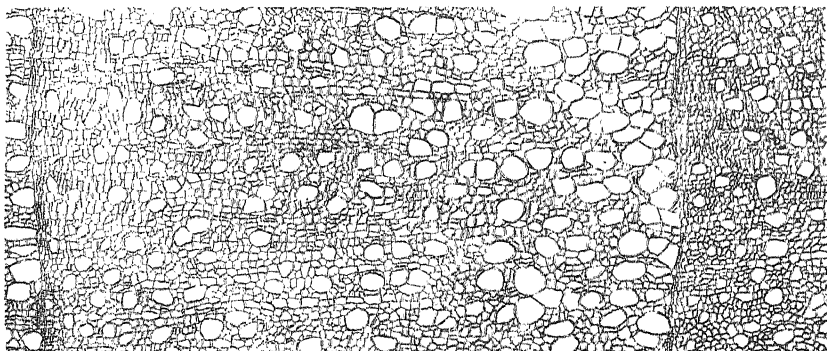
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Projection drawings of cross-sections of secondary xylem

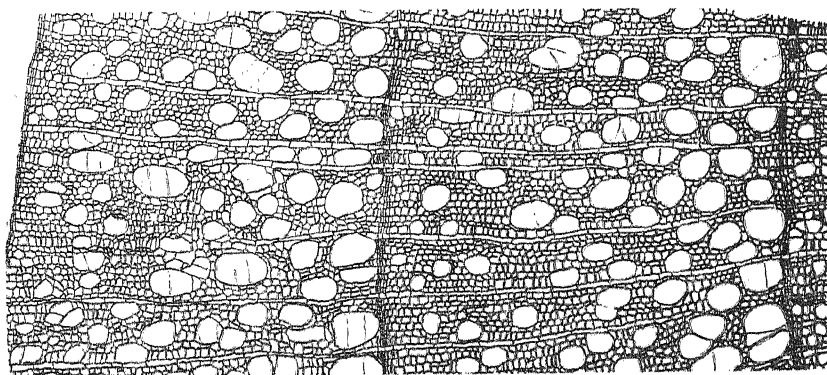
FIG. 1. Crab-apple (*Pyrus malus*).

FIG. 2. Dogwood (*Cornus stolonifera*).

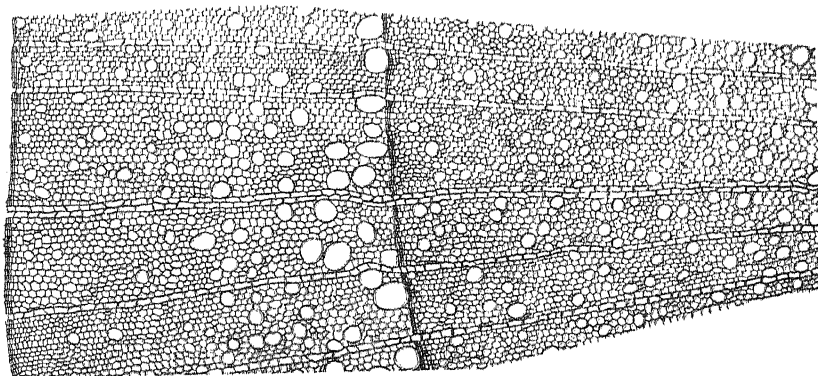
FIG. 3. Unknown variety of apple (*Pyrus malus*).



4



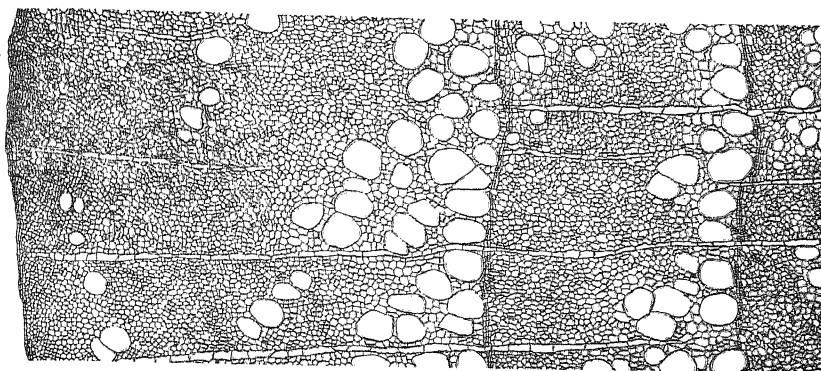
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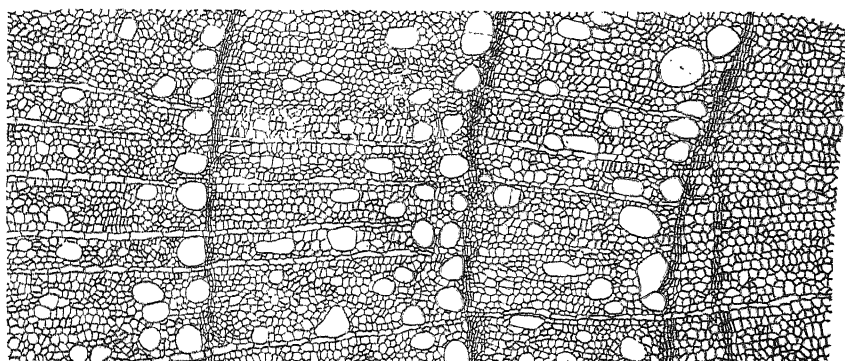
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Projection drawings of secondary xylem  
FIG. 4. Honeysuckle (*Lonicera* sp.).

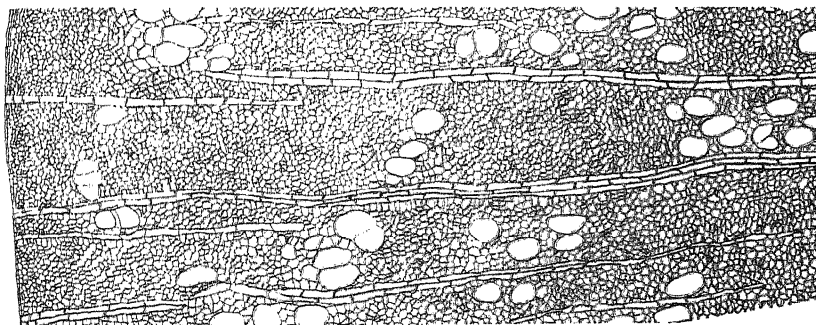




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8



9

Projection drawings of secondary xylem

FIG. 7. Russian pea (*Caragana arborescens*).

FIG. 8. Poplar (*Populus tremuloides*).

FIG. 9. Mountain ash (*Sorbus americana*).

the ring, but there is a definite suggestion of ring porosity. The vessels of the spring wood are larger than those of the summer wood.

*Salix* and *Syringa* are representatives of the transition group—the semi-diffuse-porous group. The vessels of *Salix* (fig. 5) are more numerous and more evenly distributed than those of lilac, and very much larger. The spring wood of willow contains a great number of extremely large vessels which give it a marked degree of ring porosity. Lilac (fig. 6) is considered semi-diffuse-porous because of its scattered, small vessels in the summer wood. The late summer vessels are very small, and only a few are closer than 8 or 10 tracheids to the vessels of the new year ring. The tracheids are very thick-walled.

*Caragana* (fig. 7) and *Populus* (fig. 8) are definitely ring-porous, the majority of vessels being concentrated in the spring wood, with only a few in the summer wood. The total tracheal area of *Caragana*, however, far surpasses that of *Populus*, and there are some groups of vessels which actually border on the new annual ring; for these reasons *Caragana* has been placed ahead of *Populus* in the chosen order. Also, *Caragana* is a shrub which produces a large, fast-growing annual ring, whereas *Populus* is a tree which produces smaller annual rings.

Mountain ash (*Sorbus*, fig. 9), for which there are no analytical data, is placed last in this grouping. There is in the xylem of this plant no special region of tracheal concentration; but, instead, the tracheae appear in a limited number in small groups, which are located in the spring and summer wood. The tree is a rapidly growing one, producing a large annual ring (note fig. 9) which, however, is made up almost entirely of small, very thick-walled tracheids. The resistance offered to a laterally moving stream would be extremely great, and this fact, coupled with the adverse conditions under which these plants grow, permits of little or no accumulation of salts above the girdle; in fact, in all but one of the girdled stems, death by desiccation resulted in the portion above the girdle.

A further consideration of the data of tables I and II will reveal consequences of an excessive transpiration rate. The effects of high transpiration and lack of water working together can be noticed in the data by the general tendency of the end controls to have higher ash and total nitrogen percentages than the girdled stems. This is especially true of those plants which remained growing until July 8, which indicates that, as the summer and increasing drying continued, the girdle as it affects the xylem became the limiting factor in transport of solutes up the stem. These data, then, show two important facts: (1) that the total amount of solute passing the girdle has the xylem anatomy as the limiting factor; and (2) that the great transpiration rate working in conjunction with an insufficient water supply, forces the girdle to become even more the limiting factor in the transport

of water with its solutes. These facts can be demonstrated in the first case by comparing the beginning controls with the girdle, and in the second case, by comparing the girdled stems with the end controls. It is in this second case that drought and transpiration become the forces at play on the system.

As though paradoxical to the previous data, the end controls of the diffused porous plants with thin-walled tracheids and vessels (with the exception of *Lonicera*) showed the greatest increase of ash and total nitrogen over the girdled stems when compared with the semi-diffused and ring-porous plants. The series is reversed, with the end control of *Populus* showing the least increase in ash and total nitrogen over the girdled stem. However, just the proximity of the thin-walled vessels to the exposed area increases the likelihood of their being dried out, plugged, or disrupted by the intake of gas into the region about the girdle, thus incapacitating a considerable percentage of the area of the ascending water columns. In such ring-porous woods as *Populus* and *Caragana* by far the greater portion of the conducting area lies deep in the annual ring; and any plugging of the tracheids in the girdle region will result in very little decrease in the extent of translocational facilities of the plant. The thickness of the exposed cell walls will undoubtedly be a factor determining the extent of drying or of the intake of air into the xylem elements. Thus one would expect a greater percentage of reduction of upward movement in those plants whose xylem would *normally* facilitate upward movement of solutes, and a lesser percentage of reduction in the movement of solutes in the ring-porous woods.

There is, as was pointed out above, a direct correlation between the effect of the girdle and the conditions under which the plant is growing, a fact which can be illustrated by a comparison of *Lonicera* and *Cornus*. These possess very diffuse-porous wood with vessels in large numbers bordering on the cambium or on the spring wood of annual rings, and yet the results were quite different. Their respective ash contents are as follows:

	Girdled	End control	Difference
<i>Cornus</i> .....	108.9 mg.	436.9 mg.	328.0 mg.
<i>Lonicera</i> .....	233.2 mg.	297.4 mg.	64.3 mg.

The difference may be accounted for by the difference in conditions of growth of the plants. It will be remembered that the stems of *Lonicera* were harvested on May 27, less than 6 weeks after girdling, whereas the stems of *Cornus* were left on the plant until July 8. It has been stated that most of the rainfall of the region comes in the winter and spring. The month of May is usually the last to receive much rain, and the air is humid, the soil saturated as a result of winter snows and early rains. Conditions for growth in April and May undoubtedly parallel those of a normal mesophytic tree-bearing region, so it is to be expected that results obtained in this period for *Lonicera* are more likely to approach the results which would be obtained

under normal tree-growing conditions. The effect of the girdle would, under the latter conditions, be much less pronounced than that observed for the July collection presented here. It was noticed in July that the leaves of the *Lonicera* from which the material had been collected in May were drying out and curling, a consequence of desiccation. The differences between the end controls and the girdled shoots, therefore, appear to be larger than would be the case under more humid conditions.

The data indicate that dogwood A and dogwood M, although of the same species, reacted differently to the girdle. Their places of growth are quite different, however, dogwood A being on the campus, and exposed to sunlight intensity characteristic of the region; while dogwood "M" grows against the east wall of a campus building, and is exposed part of the day to the sun, the intensity of which becomes much greater than is normal by reflection from the wall. The intense heat increases the transpiration rate to a much greater degree than in the case of dogwood A, and because of this increased transpiration the deleterious effect of the girdle is greater.

It seems, therefore, that the differences between the end control and the girdled shoot may be considered to measure the effect of the girdle on the specific limiting factor which retards the growth of the particular plant. In some cases (*Cornus*, etc.) this seems to be high temperature; in some, water, (*Pyrus*); in some lack of nitrogen in the shoots (*Populus*, etc.). It is reasonable to suppose that, were all conditions ideal for each individual species, the difference between the girdled shoots and the end control would be considerably reduced (*Lonicera*); but only in the unusual case (*Pyrus*) would the girdled shoot exceed the ungirdled one in growth.

### Summary

1. Data for girdling experiments are given for poplar (*Populus tremuloides*), willow (*Salix* sp.), dogwood (*Cornus stolonifera*) growing in two different localities, mountain ash (*Sorbus americana*), Russian pea (*Caragana arborescens*), lilac (*Syringa vulgaris*), honeysuckle (*Lonicera* sp.); and the fruit trees, apple (*Pyrus malus*, variety unknown) and crabapple (*P. malus*).

2. Shoots from each of these plants were selected in groups of three, one of these girdled, one removed at the time of girdling, and the third removed at the time the girdled shoot was removed. The difference in ash and nitrogen contents between the first and second shoots indicates the amount of each of these materials which passed the girdle; and the difference between the first and third shoots indicates the effect which the girdle, through its influence on the sum total of growth factors, imposes on the upward movement of the transpiration stream and its salts.

3. The data so obtained show, first, that a considerable amount of both

ash and nitrogen passed the girdle, thus further establishing the generalization that the upward movement of inorganic solutes takes place chiefly in the xylem; second, that the influence of the girdle is somewhat characteristic of the species and of the environment in which the plant is growing.

4. The effects of the girdle on the development of the parts above it seem to be as follows: (1) The removal of a ring of bark prevents the development of new xylem at that point, which, as growth above continues, becomes a constriction in the cross-sectional area of the conducting channel. (2) No matter how carefully a ring of bark may be removed even though no mechanical injury has been inflicted, and even though the wound is carefully covered, the xylem is affected in two ways: first, its living cells die as a result of their isolation from the living cells of the phloem; and second, as a consequence of this and of the actual exposure of the xylem, the outer layers of conducting cells lose their capacity to conduct water, and thus further limit the capacity of the xylem to conduct water with its dissolved salts. (3) The imposed girdle, as a result of the effects mentioned, makes necessary a transfer of water from one annual ring to another. The number, size, and distribution of the tracheae in the annual ring of wood seems to determine the rate with which the water with its dissolved salts can thus be transferred. A good correlation was found between tracheal distribution within the xylem of the plants studied and the amounts of minerals which passed the girdles.

5. It appears therefore that the girdle, as a break in the continuity of the phloem, does not affect the upward movement of salts; but rather that the girdle, as it affects the movement of water, influences the upward movement of salts in the xylem.

DEPARTMENT OF BOTANY  
UNIVERSITY OF HAWAII  
AND  
DEPARTMENT OF BOTANY  
UNIVERSITY OF CHICAGO

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# EFFECT OF LIGHT ON CO<sub>2</sub> IN LEAVES

JOHN SHAFER, JR.<sup>1</sup>

(WITH THREE FIGURES)

## Introduction

Recently, in an attempt to find some correlation between the average stomatal aperture of a leaf and the amount of carbon dioxide in that leaf, the author developed a piece of apparatus with which to extract and analyze the gas in a single leaf. In time it became apparent that no correlation between stomatal aperture and carbon dioxide was being found. At about the same time it began to appear that darkness is associated with a decrease in the amount of carbon dioxide in leaves. Even before this it had become obvious that leaves contain large amounts of carbon dioxide when they are in bright light. The latter part of the research was turned to the apparently new study of light effect on the carbon dioxide contents of leaves.

The extraction of gases from the leaf was performed essentially in the way followed by MAGNESS (6). The analysis of gases obtained followed the basic principles employed in any of several common volumetric gas analysis apparatuses. The analysis part of the apparatus approaches most closely, perhaps, that of BONNIER and MANGIN (2, p. 377).

Most of the work leading to the data in this paper was done on the broad bean, *Vicia faba* L. This was a very satisfactory plant because of its opposite, glabrous leaves. The following plants also were used at one time or another: nasturtium, *Tropaeolum majus* L.; beet, *Beta vulgaris* L.; wandering Jew, *Zebrina pendula* Schnizl.; red kidney bean, *Phaseolus vulgaris* L.; a variegated geranium, *Pelargonium hortorum* Bailey; a willow, *Salix* sp.; two varieties of roses, *Rosa* spp.—a white-leaved kind, "Silver Wedding Rose" (furnished by the courtesy of the Albert F. Amling Co., of Maywood, Illinois), and a green-leaved variety, "Senior."

The present research has been based on determinations of the amounts of carbon dioxide which could be extracted from leaves by a Torricellian vacuum. Nearly always the leaves have been used singly, each determination giving the amount of carbon dioxide in the gases obtained from one leaf (or leaflet, in the case of *Vicia*).

## Apparatus

The samples have been extracted and analyzed in the apparatus shown in figure 1. The flask Q holds an aqueous solution of 30 per cent. sodium hydroxide. Before the apparatus is used, the sodium hydroxide is drawn through the capillary tube P, of 1.5-mm. bore, and the stopcock M, of 1-mm.

<sup>1</sup> The writer held a Cramer Fellowship from Dartmouth College during this research.

bore; then the stopcock is closed. This arm ( $N,P,Q$ ) of the system is to be used in the analysis and must be completely filled with sodium hydroxide. Tubes  $P$  and  $N$  are joined, the end of each having been ground flat, by a short rubber tube.

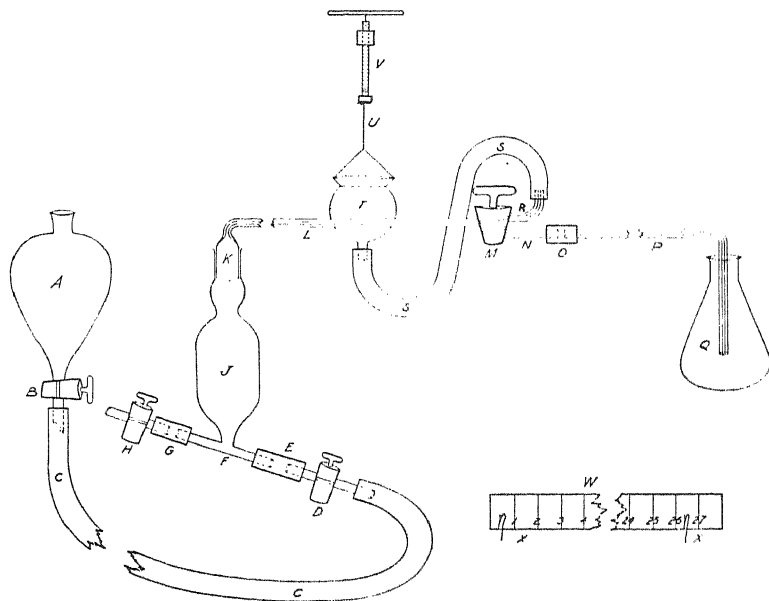


FIG. 1. Apparatus for extraction and analysis of gas from leaves.

Now the capillary tube  $L$ , of 1.5-mm. bore, must be washed and dried. This tube must be washed frequently. It is easily done by separating the ground glass joint  $K$  and applying suction to that end of tube  $L$ . Water can then be drawn through tubes  $L$  and  $S$ , followed by alcohol and then by air until the tube  $L$  is dry.

The entire apparatus, save only the arm  $N,P,Q$ , must be dry when used. With care all water can be kept from passing joint  $K$  into chamber  $J$ ; hence this need never be dried. The apparatus is next filled with mercury from the mercury reservoir  $A$  to the stopcock  $M$ . This stopcock is closed and the reservoir  $A$  lowered to produce a Torricellian vacuum. Rubber tube  $C$  must be nearly 1 meter long. Several successive evacuations are needed to free this part of the apparatus of air. All air must be removed from tube system  $G,H,D,C$ . It has been impossible to reduce the air below 0.5 cm. (Throughout this paper gas measurements are expressed in centimeters, having been measured always at atmospheric pressure).

Once freed of air, the apparatus is ready to use. The joint  $K$  is separated and the mercury allowed to drain partially from chamber  $J$ . Chamber  $J$  is completely emptied only when under vacuum; only in this way can air



be kept from going below cross-tube *F*. Into the partly empty chamber is introduced a leaf. Then the joint *K* is quickly closed and mercury is driven in to fill chamber *J*, tubes *L* and *S*, and half of levelling tube *T*. Stopcock *M* is then closed and the reservoir *A* lowered. As the chamber *J* empties, various gases quickly emerge from the leaf. After half a minute or so the mercury is returned to chamber *J*, driving the gases extracted from the leaf into tube *L*. No more attention need be given to the leaf; it will not give off any gas so long as it is covered by mercury at atmospheric pressure, and even if some were produced, it could not mix with the sample being analyzed.

For analysis the gas must be measured at some constant pressure. Atmospheric pressure is the easiest pressure to attain and is reasonably constant. Stopcock *D* is closed and that at *M* opened. The pressure is then adjusted by screwing the levelling bulb *T* up or down. The scale *W* is at this time hooked onto tube *L*. The scale used in the present work was 28 cm. long and was divided into millimeters. With a hand lens of about 7 $\times$  power one can read the mercury meniscus to tenths of millimeters with an accuracy of  $\pm 0.01$  cm. The gas bubble is most easily measured by taking the difference between the readings of its two ends.

Once the length of the bubble is known, most of the mercury between the bubble and the 3-way stopcock *M* is forced into the tube system *S-T*. Then the stopcock is reversed, and the rest of the mercury and all of the gases are allowed to pass into the tube system *N-Q*. It is best to stop in the stopcock *M* the mercury following the gas. The presence of the mercury globule in front of the gas bubble has several advantages and is quite necessary. It leaves in the tubes *N* and *P*, however, enough hydroxide solution to absorb all of any bubble introduced into those tubes, even if the bubble is pure carbon dioxide. This has been determined experimentally. Experiment has also shown that all of the carbon dioxide is removed from a sample within thirty seconds after the last of that sample has entered the hydroxide-holding tube. Therefore, at the end of half a minute the gas is drawn back into tube *L* and its length determined as before. The difference between the former and this latter bubble length is, of course, a measure of the carbon dioxide originally present. This second reading completes a determination. Several centimeters of the hydroxide solution are drawn from tubes *N* and *P* to remove mercury and exhausted sodium hydroxide. Finally the system from *K* to *T* is washed and dried, preparatory for another analysis.

Certain experimental difficulties which have been met are these: (1) Some parts of the apparatus (especially the stopcock *M*) may trap part of the sample. (2) The leaf may in one way or another trap large amounts of air, thus making the sample so large that part of it must be discarded. (3) Volume changes of the sample may be large—changes due to pressure and room temperature are slow and can be disregarded as regards single analyses. The

greater variations due to these causes over a period of days will tend to equalize each other. The proximity of the operator's body to the apparatus will cause rapid temperature, and therefore volume, changes. (4) Occasionally tube *L* and stopcock *M* become so dirty that they must be cleaned; this is easily done with aqua regia. (5) After a period of use, the stopcock *M* allows some of the sodium hydroxide solution to pass and must be greased again. (6) Some leaves, *e.g.*, those of *Vicia*, take mercury into their intercellular spaces, thereby causing the gas bubble to move toward the chamber *J*.

Various experiments to test the accuracy of this gas analysis indicate that the maximum error for any one reading is  $\pm 0.10$  cm. Only rarely does the error for any one reading exceed this value.

The bore of capillary tube *L* must be nearly constant, if one is to get accurate readings. The bore of the tube used in the present work was tested in this way: a globule of mercury about 1 cm. long was introduced into the tube and measured between each two adjacent centimeter marks on the scale from one end of the tube to the other. This was repeated ten times. Then the ten readings for each centimeter region were grouped and averaged. These average values were used in constructing the graph of figure 2. The value of  $\sigma$  was calculated for each of the average values:<sup>2</sup> the highest  $\sigma$  was 0.0217, the lowest 0.0159, the average 0.0183. Note in figure 2 that a mean

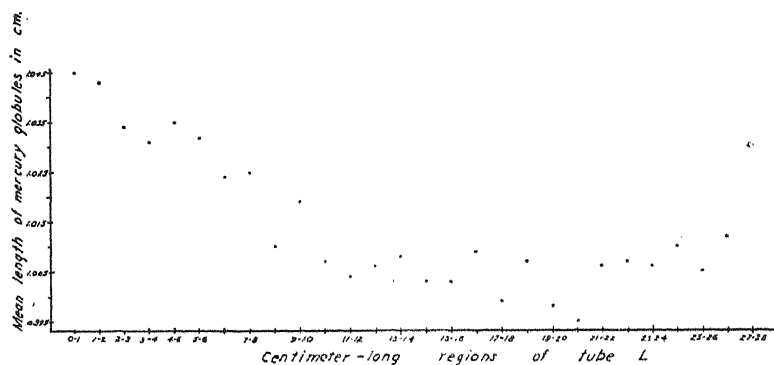


FIG. 2. Graph showing variations in capillary tube *L*.

value of  $1.005 \pm 0.10$  cm. includes most of the length of the capillary tube. The ordinate values in the graph mean nothing as absolute lengths, but they undoubtedly show the relative cross-sectional areas of the different regions of the tube with considerable accuracy.

Whether the bore of tube *L* is constant enough for use must finally be determined by the accuracy of the readings which are obtained in actual analyses. The work reported in this paper can at best have been only semi-

<sup>2</sup> Thanks are due to Professor J. R. LIVERMORE for help with the statistical part of the problem.

quantitative. Therefore, the accuracy of the apparatus may be considered sufficient if the errors do not completely obscure the results.

### Methods of analysis

Two methods have been used to test the working accuracy of the apparatus. The one employed the analysis of samples of carbon dioxide-enriched air. The other employed the analysis of numerous samples of atmospheric air taken from the laboratory.

The results of the tests employing carbon dioxide-enriched air are summarized statistically in table I. The method used was to enclose in a flask

TABLE I  
STATISTICAL SUMMARY OF DATA FROM ANALYSES OF CO<sub>2</sub>-ENRICHED AIR\*

DATE	Av. CO <sub>2</sub>	N	$\sigma_M$	ERROR OF SINGLE OB- SERVATION	% $\times$ N
				%	
3/16/36 .....	0.40	15	0.102	25.4	381.0
3/17/36 .....	0.30	15	0.082	27.3	409.5
3/19/36 .....	0.33	10	0.065	19.7	197.0
3/20/36 .....	0.31	10	0.030	9.7	97.0
					50 / 1084.5
					21.7

\* On the basis of the 50 observations included in this summary, the error of a single observation is 21.7 per cent.

the enriched air and to invert the flask over one arm of a U-tube. The mouth of the flask was sealed with mercury. The free end of the U-tube was attached to stopcock *H* of the apparatus. A sample could be drawn at will by evacuating chamber *J*, then opening stopcock *H*. Ten or fifteen small samples were drawn from the flask before it had to be refilled. It was found that the gas in the flask varied in composition unless the mercury within and outside of the flask was maintained at nearly identical levels. This was done in the tests.

The accuracy of analysis was tested in another way from these same data; the average percentage of carbon dioxide in each flask was calculated from the analytical data for the various small samples. From the average percentage it was possible to calculate what carbon dioxide reading should have been obtained from each individual analysis. This assumed that the average carbon dioxide value for a flask was a correct measure of the carbon dioxide concentration. The difference between any calculated value for carbon dioxide and the corresponding experimentally determined value was taken to be

experimental error. Results falling within  $\pm 0.10$  cm. of the calculated value were obtained from 42 of the 50 analyses.

For the tests using atmospheric air, the samples were drawn directly from the laboratory air. If the concentration of carbon dioxide in that air had been 0.03 per cent., the maximum reading obtained for carbon dioxide should have been 0.01 cm. Since the error in reading each mercury meniscus was by itself 0.01 cm., ordinary air has been considered for present purposes to have no carbon dioxide. Any difference in bubble length has been assumed to be experimental error. Of the 60 analyses made, 51 were included within limits of  $0 \pm 0.1$  cm. (table II).

Data from the results of the analyses of moist and dry air are also included in table II. The general technique used was that employed in obtain-

TABLE II

RESULTS OF ANALYSES OF AIR TO SHOW VARIATIONS DUE TO RELATIVE HUMIDITY

CONDITION OF AIR USED	AV. TOTAL VOLUME	AV. "CO <sub>2</sub> " READINGS $\pm \sigma_M$	CO <sub>2</sub>	NO. OF ANALYSES	NO. OF READINGS IN LIMITS $\pm 0.1$ CM.
Untreated .....	23.681	$0.021 \pm 0.012$	%	60	51
Dried over KOH .....	22.409	$0.019 \pm 0.014$	0.086	15	13
Moist over H <sub>2</sub> O .....	20.809	$0.010 \pm 0.009$	0.046	29	28

ing samples of carbon dioxide-enriched air. Dry air was prepared by having in the flask for several days small chunks of potassium hydroxide. Moist air was prepared by having water in the flask for several days.

Of the data for dry air, limits of  $0 \pm 0.1$  cm. include 13 of the 15 readings. This is equivalent to 52 of 60 readings, and compares well with the 51 of 60 readings obtained by the application of the same limits to the data concerning ordinary air. Of the data for moist air, the same limits include 28 of 29 readings, equivalent to 56 of 60. This, too, is close to the figure obtained from the data for ordinary air.

The apparent complete absence of any effect of water vapor on carbon dioxide readings is extraordinary. Assuming a pressure of 760 mm., a temperature of 20° C., and a 10 M. (40 per cent.) sodium hydroxide solution 70 per cent. ionized, one can calculate that a 28-cm. sample, with no carbon dioxide but with 100 per cent. relative humidity, should show a decrease after absorption of 0.17 cm. This is above the limit of error for an individual reading and should affect the results of a series of readings; yet it seems to have had no effect. Perhaps the methods of changing the humidity of the air were not effective.

Even if the presence of water vapor does affect individual readings, it will not change any final conclusions because all of the present research has been done on leaves; and the amounts of water vapor given off by the various leaves, though it may vary, is without doubt so nearly constant that any error due to the absorption of water vapor by the sodium hydroxide will appear uniformly in all analyses. The relative effect of such an error will, therefore, be zero.

In summary, it seems that the apparatus used in the present research was accurate within limits of  $\pm 0.1$  cm. for any single reading, or perhaps less, if the statistically obtained error of 21.7 per cent. is correct (table I). A summary of all the analyses of carbon dioxide-enriched air, ordinary air, dry air, and moist air shows that limits of  $\pm 0.1$  cm. include 134 of 154 analyses, or 87 per cent. of the total. The accuracy of the average of a series of readings is undoubtedly greater than  $\pm 0.10$ ; it may be greater than  $\pm 0.02$ , to judge from the various  $c_m$ 's which have been calculated. The readings are probably unaffected by any variation in the amount of water vapor (table II).

### Experimentation

Throughout the work it has been obvious that illuminated leaves contain much carbon dioxide. Experiment 1 of table IV shows data from the analyses of pairs of *Vicia* leaflets and will illustrate the point. Each member of every pair was in light and the two analyses were made in rapid succession. The average amount of carbon dioxide in the first leaflets of the various pairs was 0.322 cm., or 1.53 per cent. of the average total volume of the samples. The value for the second leaflets of the pairs checks well with this, being 0.323 cm. of carbon dioxide on the average, or 1.51 per cent. of the average total volume. Since the gas samples were contaminated by air which had been adsorbed by the surfaces of leaves and apparatus, and perhaps also by air from small leaks, and since these contaminations increased the volumes of the samples without affecting measurably the amounts of carbon dioxide in them, the percentages just given are not percentages of carbon dioxide in the gases actually obtained from the leaves. The percentages in

TABLE III  
AVERAGE VALUES OF CO<sub>2</sub> OBTAINED ON 2/27/36-2/28/36

PLANT	DAY		NIGHT	
	cm.	%	cm.	%
<i>Tropaeolum</i> .....	0.31	1.35	0.28	1.20
<i>Vicia</i> .....	0.35	2.04	0.22	1.26
<i>Beta</i> .....	0.70	2.89	0.48	2.00

the leaves must have been even higher than these figures indicate. In any case it is to be seen from these data that the vacuum method used draws much carbon dioxide from leaves, even when they are brightly lighted.

From table III it appears that there is not more extractable carbon dioxide in leaves at night than in the daytime. The data for this table were collected over a period of twenty-four consecutive hours and should therefore be comparable. Data obtained after daybreak on 2/28/36 are not included in this summary, for it is possible that the leaves must be exposed to light for a period of time before they gain more carbon dioxide.

These data suggest, indeed, that leaves contain less carbon dioxide at night than in the daytime, but by themselves are too few to prove this. However, later and more extensive work with *Vicia* makes the point clear.

To determine definitely the effect of darkness on carbon dioxide content of leaves, *Vicia faba* was used. Its paired leaflets made it especially suitable. One leaflet of a pair was analyzed at the time the plant was darkened, the other after the plant had been dark for the desired length of time. As a check on the method several pairs of leaflets were analyzed without having been subjected to any dark treatment, the second leaflet being analyzed as soon as possible after the first analysis. These data are given in table IV, experiment 1. The odds in favor of any significant difference between the

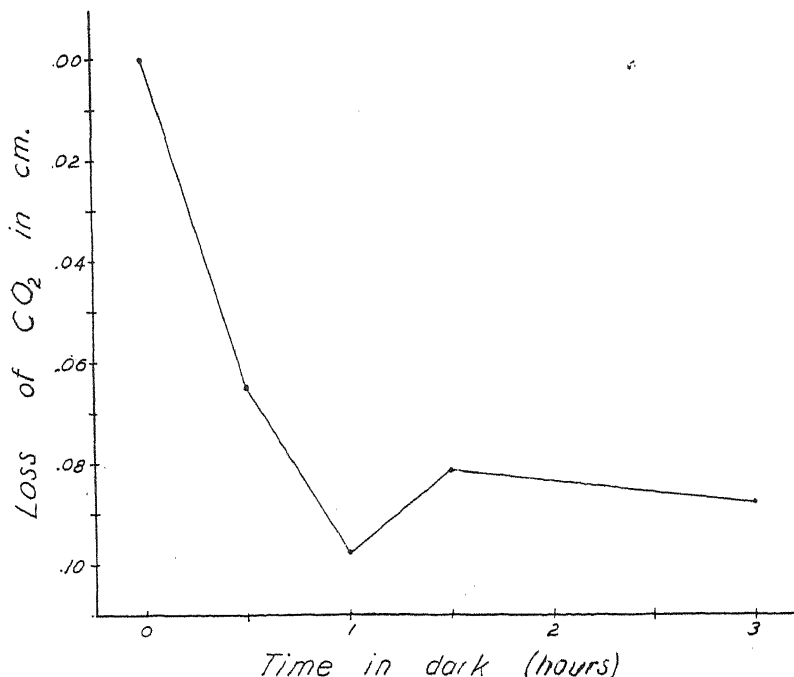


FIG. 3. Graph showing loss of CO<sub>2</sub> in leaves of *Vicia* during periods of darkness.

TABLE IV  
 PAIRED DATA FOR *Ficia* TO DEMONSTRATE EFFECTS OF DARKNESS ON CO<sub>2</sub> IN LEAVES

EXPERIMENT	IN LIGHT			TREATMENT	AFTER TREATMENT			
	NO. OF RUNS	AV. TOTAL VOLUME	AV. CO <sub>2</sub> ± σ <sub>M</sub>	CO <sub>2</sub> *	AV. CO <sub>2</sub> ± σ <sub>M</sub>	CO <sub>2</sub> *	D <sub>CO<sub>2</sub></sub>	D/σ†
1	30	21.03	0.322 ± .03	1.53	None ½ hr. dark 1 hr. dark 1½ hr. dark 3 hrs. dark 1 hr. dark (20-5° C. above greenhouse temperature)	21.44	.001	- 0.03
2	40	19.53	0.357 ± .03	1.83		19.85	.065	2.1
3	49	22.28	0.339 ± .02	1.52		22.93	.098	3.4
4	30	18.52	0.346 ± .02	1.87		20.56	.082	2.9
5	30	16.57	0.298 ± .02	1.79		17.91	.088	4.3
6	29	18.56	0.398 ± .03	2.14		18.76	.060	1.4

\* The percentage values must not be construed as giving the percentages of CO<sub>2</sub> in the gases actually emerging from the leaves. Such percentages are undoubtedly higher than those given here. See text pages 147-148.

† When D/σ is 2.1 the odds in favor of the significance of D are 27:1.

means of the two series of data are far below 2:1, as calculated by  $D/\sigma$ . Any significant difference between the averages of the carbon dioxide contents of two series of paired leaflets of *Vicia faba* must, therefore, be due to the treatment given the plants between the times of picking of the two leaflets of each pair.

The plants of *Vicia* were darkened by being put in closed cupboards. The lengths of treatment were  $\frac{1}{2}$ , 1,  $1\frac{1}{2}$ , and 3 hours. The data obtained from these various groups of experiments are given in table IV (experiments 2-5). The results of the various groups are summarized graphically in figure 3.

The curve as drawn in this graph is of necessity only relative. It can be made nearly quantitative, but only if both light and temperature are controlled throughout the experiments employed in deducing the curve. (This is true, indeed, of all work reported in this paper.) From the present work it is clear that light influences the amount of carbon dioxide. There have been strong indications as time passed that temperature, too, affected the amount. Though the precise points on the curve cannot be considered fixed, it is reasonably certain that the general form of the curve will stand. To be sure the differences between adjacent points obtained by work on treated leaves are not significant statistically. However, it seems logical that the  $\frac{1}{2}$ -hour point should be intermediate between the 0 and 1-hour points. The attainment of equilibrium, as denoted by the slight difference between the  $1\frac{1}{2}$ - and 3-hour points, is also to be expected. That this equilibrium value should be below the zero point is supported by the data in table III, for the run of February 27-28. Here for three kinds of plants the night carbon dioxide average is less than that for the day.

In the drawing of the graph the zero point was arbitrarily assumed to be constant, and the other points were plotted as differences. That is, each check was assumed in its turn to be zero, and the difference between that check and the corresponding treated leaves was plotted directly on the graph. Such a method was necessitated by the existence of a certain amount of variation between the carbon dioxide values of the various checks.

The data obtained make it very certain that, under the conditions involved in these experiments, less carbon dioxide can be extracted from *Vicia* leaflets by a vacuum after those leaflets have been in the dark half an hour or more than can be extracted from similar leaflets in the light. In every case studied, the difference between the carbon dioxide contents of lighted and darkened *Vicia* leaflets was statistically significant. That such a decrease in extractable carbon dioxide occurs in other plants is indicated by the data from the 24-hour run involving *Tropaeolum*, *Vicia*, and *Beta*; by the work on the white leaves of *Salix* and *Rosa*; and by the work on white and variegated leaves of *Pelargonium*.

In all of the *Vicia* experiments just mentioned the darkened plants were



without doubt several degrees cooler than the lighted ones. This made it impossible to say whether the effect was due to change in light or in temperature conditions. An attempt was made to separate these two by putting the plants for 1 hour into a dark chamber which was 2°–5° C. warmer than the greenhouse. The temperature of lighted leaves (not in direct sunlight), as measured by a thermocouple, was not more than 1°–2° C. above the greenhouse temperature. Therefore, the darkened leaves were in this case probably warmer than the lighted ones. Nevertheless, there was a decrease in extractable carbon dioxide when the plants were darkened (experiment 6, table IV). Unfortunately, this decrease is not statistically significant. Neither is it as large as the decrease in the earlier lot of 1-hour darkened leaves. Its existence suggests very strongly, however, that part of the decrease in carbon dioxide in the earlier experiments, if indeed not all of it, was due to a change in light conditions.

Reference has been made to experiments involving white leaves. Three kinds of plants, *Salix*, *Rosa*, and *Pelargonium*, were used in these experiments. The *Salix* leaves were white from lack of some nutrient element, probably iron. The *Rosa* and *Pelargonium* leaves were genetically white. Too few white leaves were available to provide indisputable results. The indications are very strong, however, that the carbon dioxide content of white leaves decreases in the dark just as does that of green leaves. The data from these white-leaf experiments are summarized in table V. The data found there for green leaves were collected as checks on the white-leaf data, since no work had been done previously with any of the three species concerned.

The supplies of *Salix* and *Rosa* leaves were entirely too small to furnish statistically satisfactory results. It is interesting, however, that the white leaves of each showed a greater decrease of extractable carbon dioxide in the dark than did the corresponding green leaves.

The work with *Pelargonium* indicates strongly that the carbon dioxide contents of white and of green (in this case, variegated, but mostly green) leaves are the same and are affected similarly by light and heat. This is rather evident from table V, but is more striking when it is realized that in the white-leaf average for carbon dioxide in darkened leaves, is included one abnormally high reading of 1.07 cm. If this one reading is omitted,  $D_{CO_2}$  becomes 0.071, which is nearly the same as the value for mostly-green leaves;  $D/\sigma$  becomes 1.97, which gives odds of 19:1 in favor of the significance of  $D_{CO_2}$ . Lack of more white leaves of *Pelargonium* prevented further work of this sort.

As can be seen from table V, nearly twice as much gas was obtained from the white leaves of *Pelargonium* as from the green ones, yet the amounts of carbon dioxide were about the same. The larger total volumes of the white leaves were undoubtedly correlated with the leaf structure. The white leaves

TABLE V  
EFFECT OF CHLOROPHYLL ON CO<sub>2</sub> ABSORPTION BY LEAVES

KIND OF PLANT	IN LIGHT			AFTER 1 HOUR IN DARK			
	NO. OF RUNS	AV. TOTAL VOLUME	AV. CO <sub>2</sub> ± σ <sub>M</sub>	CO <sub>2</sub> *	AV. TOTAL VOLUME	AV. CO <sub>2</sub> ± σ <sub>M</sub>	D <sub>CO<sub>2</sub></sub>
<i>Salix</i>				%		%	
White .....	6	10.01	0.122 ± .02	1.2	10.66	0.093 ± .03	0.029
Green .....	6	10.21	0.190 ± .05	1.9	9.03	0.198 ± .03	- 0.008
<i>Rosa</i> †							
White .....	9	12.11	0.450 ± .06	3.7	13.68	0.354 ± .05	0.096
Green .....	6	16.59	0.508 ± .06	3.1	15.49	0.503 ± .07	0.005
<i>Pelargonium</i> ‡							
White .....	26	19.88	0.347 ± .02	1.7	20.32	0.307 ± .04	0.040
Green .....	26	11.90	0.364 ± .02	3.1	13.12	0.297 ± .03	0.067

\* Percentage values must not be construed as giving the percentage of CO<sub>2</sub> in the gases actually emerging from the leaves. Such percentages are undoubtedly higher than those given here. See text pages 147-148.

† The dark-treated roses, white and green, were analyzed an hour or more after nightfall had darkened them naturally. Such analyses were made on the same day as, but 6-12 hours after, the analyses of rose leaves in the light.

‡ For white *Pelargonium* D/σ = 0.86; for green D/σ = 1.97.

were somewhat larger, more hairy, and distinctly more succulent than the green ones. The white ones seem to have held more air on their surfaces and in their intercellular spaces because of these structural modifications. The modifications seem not, however, to have affected the carbon dioxide-holding power of the leaves.

### Results and discussion

From the findings of this research three facts stand out. First, brightly lighted leaves contain a considerable amount of carbon dioxide which can be extracted and measured by the methods herein described; second, the amount of extractable carbon dioxide in leaves is no higher at night than in the daytime; and third, leaves lose carbon dioxide when taken from bright light and placed at once in darkness.

There has been developed a tentative theory to explain the presence of much carbon dioxide in lighted leaves, and the disappearance of some of that gas in the dark. The effect of temperature is so little known that no effort has been made to include an explanation of its action. It is obvious, however, that light is an important factor.

It seems impossible that all of the carbon dioxide found, at least 2 per cent. in some cases (table V), should exist as gas in the intercellular spaces. If the intercellular air contained 2 per cent. carbon dioxide, it would be impossible for leaves to obtain any of that gas from air, because the diffusion gradient would be in the wrong direction.

Since the carbon dioxide cannot be free, it must be bound in some way. Since a great deal of it can be rapidly extracted by a vacuum of approximately 0.01 atmosphere, it must be loosely bound. It might conceivably be bound in any of three ways: by solution, by adsorption, by loose chemical combination. It is very unlikely that the gas is in solution. A leaf weighing 0.5 gm. (and the *Vicia* leaflets used weighed only 0.2–0.3 gm.) would dissolve from air in its contained water only about 0.00015 cc. of carbon dioxide at room temperature, whereas the amount found experimentally was 50 times that much. Moreover, the decrease of carbon dioxide in the dark cannot readily be explained on the basis of solution; light should not have any pronounced effect on the solubility of a gas, and the drop in temperature occurring in the dark should increase solubility, not decrease it. It seems impossible on the basis of present data to determine which of the remaining two explanations is the better, whether the gas is adsorbed or loosely combined.

It seems probable from the present work that the material holding the carbon dioxide is either activated by light to allow it to adsorb the gas, or it is produced in the presence of light, undergoing subsequent combination with the gas. Temperature may conceivably be a supplementary agent influencing the production or activation of such a substance. In the absence of light

the material either holds the gas so firmly that a vacuum will not extract it, or more probably does not hold it at all, so that the gas is rapidly lost by the leaf.

The curve in figure 3 suggests that there are at least two factors affecting the amount of carbon dioxide in leaves. When a leaf is darkened, the light-activated carbon dioxide-holding mechanism releases its carbon dioxide. This occurs rapidly but not all at once. The resulting drop in extractable carbon dioxide is shown by the first and descending arm of the curve in figure 3. Respiration is constantly producing carbon dioxide, however, and after a time the carbon dioxide content of the leaf reaches a sort of equilibrium value, at which time the production of that gas is just balanced by its loss. The assumption is that then the dark-acting carbon dioxide-holding mechanism of SPOEHR and MCGEE (8) and of others is saturated. (SPOEHR and MCGEE used dried leaf material, and found that when moistened it would absorb much carbon dioxide in the dark.) It is difficult to see why the amount of carbon dioxide should become temporarily less than that in equilibrium with respiration. Perhaps the apparent minimum is due only to experimental error. If, however, the carbon dioxide is held more tightly in the dark, rather than being freed, the minimum may be a true one. If so, the subsequent rise represents the accumulation of carbon dioxide from respiration, until finally the equilibrium value is reached.

The most obvious assumption in connection with the light-activated absorption of carbon dioxide is that it depends in some way on chlorophyll. It was to test such an assumption that the white-leaf experiments were performed. As stated on page 151, the carbon dioxide contents of white leaves seem subject to the same variations under the same conditions as are the contents of green leaves. Hence, chlorophyll is not a factor concerned in the light-activated absorption of carbon dioxide by leaves.

This increased carbon dioxide content may be connected in some way with photosynthesis. On the other hand, it is conceivable that the carbon dioxide comes from an intermediate unstable product of respiration, whose formation is induced by light.

Few papers dealing with the extracted gases of leaves have been found. That by BOUSSINGAULT (1) does not give data in any such form that they can be compared with those presented here. The papers by GRÉHANT and PEYROU (3, 4) and by PEYROU (7) are pertinent, but difficult of interpretation. Their work seems to be the only extensive study devoted to the gases actually inside of leaves. Some of their data support the work here reported; other data contradict it. Probably their data cannot be considered very seriously either in support or in contradiction of this work.

Two factors make it difficult to compare satisfactorily their earlier experiments with these recent ones. For one thing, there seems considerable doubt

as to the accuracy of the work of GRÉHANT and PEYROU. Their extraction was slow, and involved the heating of the leaves. These factors together probably caused respiration to influence markedly the composition of the gases obtained, concealing completely the original O<sub>2</sub>/CO<sub>2</sub> ratio. Carbon dioxide frequently formed 50 per cent. of the gases.

Assuming that the method was exact, however, one meets great difficulty in interpreting the data. For the most part, the experiments were not paired, they were not run in rapid succession, and they did not even use leaves from the same plant. Moreover, when obtained, the data were calculated in such a way as to emphasize the oxygen variations, and not those of carbon dioxide.

Another paper with possible significance in regard to the work just reported was written by KOSTYTSCHEW (5) in 1921. He reports that leaves put into chambers with high carbon dioxide concentration (6–10 per cent.) show at first a high CO<sub>2</sub>–O<sub>2</sub> ratio. Later, if photosynthesis is rapid, this drops to approximately 1. He suggests “dass eine bedeutende CO<sub>2</sub>-Menge ohne entsprechende Sauerstoffausscheidung chemisch gebunden war.” Later he says, “Höchstwahrscheinlich steht die hervorgehobene Tatsache im Zusammenhange mit der wichtigen Beobachtung von WILLSTÄTTER und STOLL hinsichtlich der CO<sub>2</sub>-Bindung durch kolloidale Chlorophyllösungen.”

In light of the work reported in the present paper, it seems very likely that KOSTYTSCHEW was dealing with the light-activated absorption of carbon dioxide. This is suggested by (1) the preliminary large absorption of carbon dioxide, (2) the lack of such absorption in darkened leaves (only two cases given), and (3) the release of most of this carbon dioxide only if the partial pressure of the gas became rather low. Although they support and supplement the work of the writer, the data presented by KOSTYTSCHEW cannot be considered definitely to prove the correctness of the present work.

### Summary

A method has been developed whereby the gases in a single leaf can be extracted and analyzed for carbon dioxide. By the use of this certain data have been obtained, and from these data the following conclusions have been drawn.

1. Leaves in light or in dark contain much carbon dioxide.
2. Leaves in the light contain more carbon dioxide than those in the dark.
3. Carbon dioxide gas is not entirely or mainly in the intercellular spaces.
4. This gas cannot be held simply in solution.
5. Part of the carbon dioxide in lighted leaves is held by some light-activated mechanism which involves adsorption or loose chemical combination.
6. Chlorophyll is not concerned with this light-activated mechanism.

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CORNELL UNIVERSITY

ITHACA, NEW YORK

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# EFFECT OF HYDROGEN-ION CONCENTRATION ON *CHLORELLA* PHOTOSYNTHESIS

ROBERT EMERSON AND LOWELL GREEN  
(WITH TWO FIGURES)

## Introduction

Aquatic green plants can assimilate carbon dioxide in solutions of widely varying hydrogen-ion concentration. Our purpose in investigating the rate of photosynthesis as a function of this factor was to clarify the interpretation of experiments in carbonate mixtures, where the concentrations of carbonate and bicarbonate ions and of free carbon dioxide all vary with the hydrogen-ion concentration. The relationships are shown in figure 1, where the percentage of total carbon dioxide in each of these three forms is plotted against the pH. The range covered by the carbonate mixtures is to the right of pH 8. From here the proportion of bicarbonate, which is at first maximal, declines with increasing pH, and the carbonate, extremely low at first, rises gradually to its maximum somewhere beyond pH 12. At the same time the proportion of free carbon dioxide declines

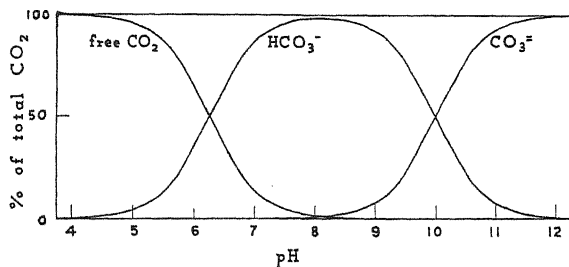


FIG. 1. Proportion of total carbon dioxide in each of the three forms, free carbonic acid ( $\text{CO}_2 + \text{H}_2\text{CO}_3$ ), bicarbonate ions, and carbonate ions as a function of hydrogen-ion concentration.

from its already very low value at pH 8, and becomes insignificant at pH 9. Nevertheless, if the carbonate and bicarbonate concentrations are high enough, the concentration of free carbon dioxide remains appreciable, for purposes of measuring photosynthesis, down to pH 11. If the mixtures are prepared by mixing M/10 solutions of carbonate and bicarbonate, then at pH 9 the concentration of free carbon dioxide will be sufficient to "saturate" photosynthesis, that is to say, cells in this concentration of carbon dioxide will show the maximum rate of photosynthesis of which they are capable. This rate will be independent of even relatively large changes of concentration. At pH 10, obtained according to figure 1 by mixing equal parts of carbonate and bicarbonate, the concentration of free carbon

dioxide will be "limiting" for photosynthesis, or so low that the rate of photosynthesis is now a function of the concentration.

The solutions made by mixing M/10 carbonate and bicarbonate have a fair buffering capacity for carbon dioxide, a considerable amount of which may be used by assimilating cells without much change in concentration. WARBURG (17) prepared a series of carbonate-bicarbonate mixtures covering the range of carbon dioxide concentrations physiologically important for green plants, and used these mixtures to study photosynthesis as a function of the concentration of free carbon dioxide, on the assumption that among the several variables in the mixtures this factor alone was significant for photosynthesis. Although this assumption has been questioned by plant physiologists from time to time, there has been a tendency to accept NATHAN-SOHN'S (10) evidence in support of it (*cf.* also BENECKE and JOST, 4, p. 201). JAMES (9) has discussed the possible influence of various factors in the carbonate mixtures, but his experimental results appear to support NATHAN-SOHN'S opinion, that the determining factor for assimilation is the concentration of free carbon dioxide. The work of OSTERHOUT and DORCAS (11) and OSTERHOUT and HAAS (12) may also be regarded as giving support to this viewpoint. However, WARBURG'S curves for *Chlorella* photosynthesis as a function of carbon dioxide concentration, and certain of the phenomena he observed in connection with temperature coefficients and the action of inhibitors, are in disagreement with results obtained by VAN DEN HONERT (7) and VAN DER PAAUW (13, 15) with *Hormidium*. In the *Hormidium* experiments the cells did not come in direct contact with carbonate mixtures, but were kept moistened with culture fluid. Both VAN DEN HONERT and VAN DER PAAUW have made several objections to WARBURG'S technique, particularly to the alkalinity of the carbonate mixtures, but VAN DER PAAUW (14), after comparing his own method with WARBURG'S, concluded that some of the disagreements in results were due to real physiological differences between *Hormidium* and *Chlorella*, and that the methods were of about equal value. Unfortunately his method was applicable only to the range of saturating carbon dioxide concentrations. The most striking differences between WARBURG'S results and VAN DEN HONERT'S were in the range of limiting concentrations of carbon dioxide, where the characteristics of photosynthesis are of particular importance because they should reveal something of the reactions entered into by carbon dioxide. But when the concentration of free carbon dioxide is varied by using different carbonate mixtures, after the method of WARBURG, the concentrations of carbonate and bicarbonate as well as hydrogen ions are subject to the variations shown in figure 1, and it is by no means certain that these three factors are without influence on photosynthesis. Recent work of ARENS (1, 2, 3) shows that some water plants may absorb both carbonate and bicarbonate ions, and he makes it appear likely that carbon dioxide absorbed in these forms can be used for assimilation.



The concentration of free carbon dioxide in the carbonate mixtures can be varied by making the various mixtures more or less concentrated, as well as by mixing different proportions of a given concentration of carbonate and bicarbonate. For a particular mixture, the pII does not vary much with the concentration, while the free carbon dioxide is roughly proportional to it. Some independent control of the several factors is possible in this way, but the available range is small because the more dilute mixtures have insufficient buffering capacity to permit accurate measurements of assimilation, and the more concentrated mixtures are unphysiological. Measurements conducted in two series of mixtures of M/10 and M/20 concentration show that for a given calculated concentration of free carbon dioxide, the rate of photosynthesis is always higher in the more dilute mixture. This suggests that other factors besides the concentration of free carbon dioxide determine the rate of photosynthesis in the carbonate mixtures. Values published by various authors for the constants required to calculate the concentration of free carbon dioxide do not agree well, and it is possible that our calculated concentrations are not quite correct, but they are probably correct relative to one another, so it is hardly possible that errors in the constants used are responsible for our results. Differences in the ionic strength or osmotic pressure of the mixtures are also unlikely to be directly responsible because *Chlorella* photosynthesis is indifferent to these factors over a wide range. The other variables are the concentrations of carbonate, bicarbonate, and hydrogen ions. Since the concentration of the latter establishes the relative concentrations of carbonate and bicarbonate ions as well as of free carbon dioxide, the importance of studying the influence of pH on photosynthesis is evident.

Although conditions in the carbonate mixtures are of special interest, they do not offer a good starting point for this investigation because of the close interdependence of all the variables under consideration. Greater freedom of control of variables is achieved by working on the acid side of pII 8, in phosphate instead of carbonate buffers. It becomes possible to establish the concentration of free carbon dioxide by saturation with a fixed partial pressure of carbon dioxide, and the bicarbonate concentration alone will vary with the pII. The carbonate ions become negligible on the acid side of pII 8. It is safe to assume that the concentrations of the two phosphate ions, by means of which the pH is controlled, are without effect on the rate of photosynthesis over the range in our experiments.

By going from carbonate to phosphate buffers we have achieved a certain simplification, but we have also added an important limitation, and turned away from our original problem. The limitation concerns the available range of carbon dioxide partial pressures at which we can study photosynthesis by the usual manometric technique. The most important feature of the carbonate mixtures is that they maintain low partial pressures of carbon

dioxide nearly constant even while considerable amounts are used for photosynthesis. It is thus possible to follow the rate of photosynthesis for long periods at carbon dioxide partial pressures far below that of ordinary air. But if acid phosphate at pH 4.6 is saturated with ordinary air, it will not contain enough carbon dioxide to make a single rate measurement. A quantity of cells sufficient to give satisfactory manometer readings will consume all the carbon dioxide in the first few moments of illumination. Therefore in experiments using phosphate buffers, we are limited to concentrations of carbon dioxide so large that the amount withdrawn by the assimilating cells makes no significant change in the total concentration. The lowest practicable carbon dioxide concentration is 0.5 per cent. The upper limit is about 5 per cent., because higher concentrations begin to have a narcotic effect on assimilation. But from 5 to 0.5 per cent. the rate of photosynthesis is independent of carbon dioxide partial pressure, while our original interest was in the range of limiting carbon dioxide concentrations. Several years' efforts have convinced us that new methods are required to investigate the influence of hydrogen-ion concentration over this range, and our attempts along these lines have not been entirely successful. We believe, however, that the simpler conditions prevailing in the phosphate buffers are of sufficient interest to justify presentation of our results, and that an understanding of the behavior of photosynthesis under these conditions should precede the investigation of the more complex relationships beyond pH 8.

### Investigation

Photosynthesis was measured manometrically in the usual way, with *Chlorella vulgaris* or *C. pyrenoidosa* cells suspended in M/25 potassium phos-

TABLE I

RETENTIONS  $\left(\frac{\Delta u}{\Delta p}\right)$  AND PH VALUES OF PHOSPHATE BUFFERS, AT 20° C.

Composition .....	Parts $\frac{M}{25}$ $KH_2PO_4$ .....	10	9	5	1
	Parts $\frac{M}{25}$ $K_2HPO_4$ .....	0	1	5	9
Before saturation .....	pH { calculated .....	.....	6.0	6.9	7.8
	colorimetric .....	4.6	6.0	6.9	7.7
Saturated with 0.5% CO <sub>2</sub> in air .....	pH, colorimetric .....	4.6	6.0	6.8	7.6
	$\frac{\Delta u}{\Delta p}$ , calculated .....	0	0.05	0.28	0.86
	pH, colorimetric .....	4.6	5.9	6.6	7.0
Saturated with 5% CO <sub>2</sub> in air .....	$\frac{\Delta u}{\Delta p}$ { calculated .....	0	0.03	0.13	0.21
	by dilution .....	.....	0.025	0.14	0.23
	by slight acidification .....	.....	.....	0.12	0.22
	by excess acidification .....	.....	0.026	0.12	.....

phate buffers. The compositions of the four mixtures used are shown in the two top rows of table I. The next two rows show the pH of each mixture before saturation with carbon dioxide in air, both calculated and determined colorimetrically with indicators. The fifth and seventh horizontal rows show the pH (determined colorimetrically) when the mixtures were saturated with 0.5 and 5 per cent. carbon dioxide in air.

The rate of photosynthesis was computed from the pressure change on the manometers, which were read at 5-minute intervals. In the potassium dihydrogen phosphate solution at pH 4.6, the bicarbonate concentration is negligible (fig. 1) and the calculation of the gas exchange is made in the ordinary way (WARBURG 18, p. 104, equation 6) from the gas and fluid volumes, the temperature, and the solubility of the exchanged gases in water. In the buffers containing dipotassium hydrogen phosphate, the dissociation of carbonic acid becomes appreciable, and the resulting bicarbonate ions alter the behavior of the solution. When carbon dioxide is used for photosynthesis, part is supplied by decomposition of the bicarbonate. The result is that a given fall in carbon dioxide pressure represents a larger consumption of carbon dioxide than if no bicarbonate were present, and another constant must be added to the expression for calculating gas exchange. WARBURG has called this the chemical "retention" for carbonic acid, and developed three methods for measuring retention in blood serum which are directly applicable to our phosphate buffers when these are saturated with 5 per cent. carbon dioxide in air (18, p. 208-212). The last three rows of table I show

retentions,  $\frac{\Delta u}{\Delta p}$  in WARBURG's notation, for our mixtures by the three different methods.

WARBURG has also shown (18, footnote p. 207) how the retention in phosphate can be calculated from the dissociation constants of carbonic and phosphoric acids. While direct measurement is to be preferred whenever possible, the calculated retentions are given for comparison (row 8, table I), directly above the experimental retentions. For each pH the retentions obtained in the different ways agree well with one another.

When the phosphate buffers are saturated with 0.5 instead of 5 per cent. carbon dioxide the bicarbonate concentrations become too low for satisfactory application of the methods for measuring retention, so for the lower partial pressure of carbon dioxide table I shows only calculated retentions (row 6). The agreement between the calculated and experimental retentions for the higher partial pressure is sufficient to give confidence in the method of calculation.

For calculating retentions, as well as for calculating the concentrations of bicarbonate ions in the phosphate solutions,  $pK'_1$  and  $pK'_2$  for carbonic acid were assigned values of 6.25 and 10 respectively, at 20° C. For calcu-

lating carbon dioxide concentrations in the carbonate mixtures, the value of  $pK'_1$  was changed to 6.22, while the value of 10 for  $pK'_2$  was retained. To select from the literature the best limiting values for the constants, and to apply the appropriate corrections for ionic strength, has entailed a number of choices which may seem arbitrary. While we would like to support our choices by a discussion of the literature, this is unnecessary for the purposes of the present paper and is reserved until a later date. For the present it is sufficient to explain that the value of  $pK'_1$  was obtained by correcting SIEDLOVSKY'S and MACINNES' (16) figure of 6.39 for  $pK_1$ , for ionic strength according to an equation used by HASTINGS and SENDROY (6). Our value of  $pK'_2$  was obtained directly from WEIDER'S (21) determinations.

Under the simple conditions of our experiments, it is safe to assume the photosynthetic quotient ( $\gamma$  in WARBURG'S equation 6, referred to above) of *Chlorella* is unity. It is therefore possible to check the retention figures by using WARBURG'S differential method (18, pp. 104-108) for the photosynthesis measurements. Each determination is made in duplicate, in two vessels of approximately equal volume, containing equal quantities of cells, but unequal volumes of fluid. If the retentions are correct, the computed gas exchange in each pair should agree.

Table II shows the rates of photosynthesis of *C. pyrenoidosa* in 5 per cent. carbon dioxide from pH 4.6 to 7. The concentrations of hydrogen and bicarbonate ions are shown in the second and third horizontal rows. The rates of photosynthesis for two different experiments (a and b) are shown

TABLE II

RATES OF PHOTOSYNTHESIS, IN CUBIC MILLIMETERS OF OXYGEN PER HOUR PER CUBIC MILLIMETER OF CELLS, CORRECTED FOR RESPIRATION, AT 20° C., FOR *Chlorella pyrenoidosa* CELLS SUSPENDED IN VARIOUS PHOSPHATE MIXTURES SATURATED WITH 5 PER CENT. CARBON DIOXIDE IN AIR. CONCENTRATION OF DISSOLVED FREE CARBON DIOXIDE IS 2 MILLIMOLES PER LITER. ROW (a) GIVES RATES FOR AN OLD CULTURE; ROW (b) FOR A FRESH CULTURE

pH	4.6	5.9		6.6		7.0	
$[H^+]$ , moles per liter $\times 10^7$ .....	250	12.6		2.5		1.0	
$[HCO_3^-]$ , moles per liter $\times 10^3$ .....	5	100		500		1200	
Volume of suspension, ml. ....	7	7	3	7	3	7	3
Rates of photosynthesis { (a) .....	12.4	12.4	11.6	12.7	12.8	12.4	13.1
	28.6	27.4	27.5	28.7	29.0	28.6	29.7

in the two bottom rows. Except for the determinations at pH 4.6, all rates were measured in duplicate with two different fluid volumes (row 4, table II). The good agreement between the duplicate determinations shows that the retention figures are fairly accurate. In spite of the wide range of con-

centrations of bicarbonate and hydrogen ions covered in this experiment, the rate of photosynthesis remains remarkably constant. The small variations are random and of no significance.

To cover a somewhat wider range of pH, a carbonate buffer of pH 8.9 was added to the phosphate series. The computed concentration of free carbon dioxide in this mixture corresponds to a partial pressure of about 0.5 per cent. carbon dioxide in air, so to maintain the concentration comparable throughout the series, the phosphate buffers were saturated with this gas mixture. The exact composition of the gas mixture is of no importance because although in the neighborhood of pH 8.5 the rate of photosynthesis varies a little with the composition of the carbonate mixture, we shall show that in phosphate it is constant over the range of carbon dioxide partial pressures covered in these experiments (see fig. 2 and accompanying discussion).

Table III shows rates of photosynthesis for two experiments covering the range of pH 4.6 to 8.9, one for *C. pyrenoidosa*, the other for *C. vulgaris*. It will be recalled that for 0.5 per cent. carbon dioxide the retentions were computed, and not measured experimentally as for table II. The agreement between duplicate determinations nevertheless remains satisfactory. Although the rates of photosynthesis are a little scattered, they show no tendency to vary consistently with the concentration of hydrogen, bicarbonate, or carbonate ions. There is therefore no indication that any one of these factors plays a part in determining the rate of photosynthesis, at least down to 0.5 per cent. carbon dioxide.

During the measurements of photosynthesis in phosphate saturated with 0.5 per cent. carbon dioxide, the latter was used up so rapidly that its concentration must have undergone considerable change even in the course of the brief rate determinations (10 or 15 minutes). It was stated above that in these experiments the exact concentration was of no importance. This was established by using a technique developed by WARBURG and KUBOWITZ (19) for the study of respiration at low partial pressures of oxygen. They admitted a small amount of oxygen to a vessel containing a suspension of respiring cells, and followed the disappearance of oxygen on a differential manometer from minute to minute until it was gone. They could then compute the concentration of the oxygen remaining at any given time, and the corresponding rate of respiration. Adapting the technique to photosynthesis, we passed known amounts of carbon dioxide into a vessel containing a suspension of *Chlorella* cells. A saturating light intensity was used for these experiments, so that, at the higher carbon dioxide concentrations, the only external factor limiting photosynthesis was temperature. The rates of photosynthesis were calculated for various carbon dioxide concentrations in the same way that WARBURG and KUBOWITZ (19) calculated respiration. For

TABLE III

RATES OF PHOTOSYNTHESIS, IN CUBIC MILLIMETERS OF OXYGEN PER HOUR PER CUBIC MILLIMETER OF CELLS, CORRECTED FOR RESPIRATION, AT 20° C., TWO SPECIES OF *CHLORELLA*, SUSPENDED IN VARIOUS PHOSPHATE MIXTURES SATURATED WITH 0.5 PER CENT. CARBON DIOXIDE IN AIR, AND IN A CARBONATE MIXTURE OF PH 8.9 IN EQUILIBRIUM WITH THE SAME CARBON DIOXIDE PARTIAL PRESSURE. CONCENTRATION OF DISSOLVED FREE CARBON DIOXIDE 0.2 MILLIMOLES PER LITER

pH	4.6	6.0	6.8	7.6	8.9
$[CO_2], \text{ moles per liter} \times 10^6$	25,000 5	1000 120	158 800	25 5000	1.3 92,000
ume of suspension, ml.	7	7	7	7	7
es of photosynthesis	12.2 11.4	10.9 11.6	13.4 12.4	13.1 11.9	12.1 10.8
	{ <i>C. vulgaris</i>		3	3	
	{ <i>C. pyrenoidosa</i>		14.9 13.5	14.0 12.7	

*C. pyrenoidosa* cells suspended in M/25 phosphate at pH 4.6, the rate of photosynthesis is plotted against carbon dioxide concentration in figure 2. Its perfect constancy over almost the entire range of carbon dioxide concentrations is remarkable, especially when contrasted with the corresponding plot of measurements made in carbonate mixtures (*cf.* WARBURG, 17, p. 254, fig. 10; EMERSON and ARNOLD, 5, p. 409, fig. 10). In these cases the upper part of the

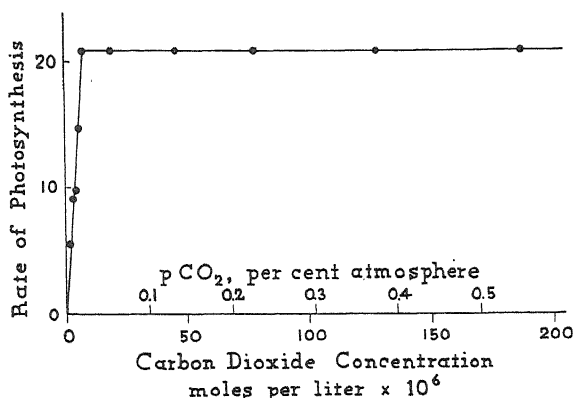


FIG. 2. Rate of photosynthesis in M/25 phosphate at 25° C. in cubic millimeters of oxygen per hour per cubic millimeter of cells, as a function of carbon dioxide concentration in the suspending fluid in moles per liter  $\times 10^6$ . Auxiliary scale on upper side of axis of abscissae shows the partial pressures of carbon dioxide in equilibrium with the corresponding concentrations. The differential manometer method described in the text was used to obtain this curve.

curve is never quite horizontal. Figure 2, although made with the same species of *Chlorella* as WARBURG's, more closely resembles VAN DEN HONERT's curve for *Horridium* (8, p. 1017, fig. 3). Our curve shows photosynthesis remaining constant even down to a slightly lower partial pressure than in his experiment. There can be no doubt that in this particular case the difference between WARBURG's results and VAN DEN HONERT's is due not to physiological differences between the organisms used, but to differences in the methods. VAN DEN HONERT's cells never came in contact with carbonate mixtures, and the conditions for his experiment were comparable with those for figure 2. The difference between WARBURG's carbon dioxide curve and our figure 2 supports the conclusion drawn earlier in this paper, from the experiments in carbonate mixtures at different concentrations, that other factors besides the concentration of free carbon dioxide determine the rate of photosynthesis in the carbonate mixtures. These mixtures do not give us a true picture of the influence of carbon dioxide concentration on photosynthesis.

The curve in figure 2 is taken from one of many attempts to measure photosynthesis in phosphate at limiting concentrations of carbon dioxide. While there is no doubt concerning the horizontal portion, which continues

straight to 5 per cent. carbon dioxide, ten times the range of concentration shown in the figure, the shape of the descending limb is uncertain because of limitations inherent in the method. It would probably be satisfactory if photosynthesis could be studied from the exchange of carbon dioxide alone, just as WARBURG and KUBOWITZ studied respiration from the exchange of oxygen alone. They achieved this by using alkali to absorb the carbon dioxide produced. In our photosynthesis experiments we have tried to use yellow phosphorus to absorb the oxygen produced, in order to measure photosynthesis from changes in carbon dioxide pressure. We were able to confirm the general belief that anaerobiosis in the dark does not interfere with the immediate start of photosynthesis when the light is turned on, but found that continued illumination in the presence of yellow phosphorus produced a slowly declining rate of photosynthesis. It is possible that the presence of phosphorus rather than the absence of oxygen was responsible for the injury, although one would be inclined to doubt this because the exposure of cells to the presence of yellow phosphorus in the dark for many hours is quite without effect on subsequent photosynthesis. In any case we were unable to obtain constant rates of photosynthesis at constant carbon dioxide concentrations if yellow phosphorus was present, and were therefore obliged to work under aerobic conditions. For experiments under aerobic conditions it is necessary to calculate the gas exchange from the combined change in pressure of both oxygen and carbon dioxide, assuming that they are exchanged in equal amounts. This is certainly a close approximation to the truth, and is entirely satisfactory for determinations made with ordinary open manometers. It is also satisfactory for the differential manometer experiments over the range covered by the horizontal portion of the curve in figure 2. But the vessel constant for the differential manometer is more sensitive to changes in the ratio of exchange of oxygen and carbon dioxide because for these experiments the fluid volume must be much smaller in proportion to the gas volume than for the ordinary manometer experiments, in order to keep the diffusion resistance as small as possible. This becomes important when the rate of photosynthesis begins to decline with declining carbon dioxide concentration because, although the photosynthetic quotient may remain perfectly constant until the last trace of carbon dioxide is gone, there are evidently other physiological processes which lead to the evolution of small amounts of oxygen in the light, and they may greatly alter the ratio of exchange of carbon dioxide and oxygen as the carbon dioxide concentration and photosynthesis approach zero. The actual amount of oxygen produced in these accessory processes is small, but sufficient to introduce considerable uncertainty into the points on the descending portion of the curve in figure 2. The chief disturbing process is nitrate reduction (*cf.* WARBURG and NEGELEIN, 20), which may continue to produce oxygen long after the last traces of carbon dioxide are gone and photosynthesis has ceased. Sometimes this



happens even with cells previously starved for nitrate for many hours, and at other times the process seems to be wholly in abeyance. We are never certain that it has not disturbed the apparent course of photosynthesis somewhat, even when the pressure change appears to cease with the disappearance of the last traces of carbon dioxide. Our differential manometer experiments will tell us down to what partial pressure photosynthesis remains constant, but they fail to show exactly how the rate changes when it becomes limited by carbon dioxide concentration.

### Summary and conclusions

For the present, then, our investigation of the influence of the hydrogen-ion concentration on photosynthesis stops at the upper range of carbon dioxide concentrations obtainable in carbonate mixtures, and leaves untouched the more interesting range of limiting concentrations. At saturating concentrations of carbon dioxide, neither the hydrogen- nor bicarbonate-ion concentration influences the rate of photosynthesis from pH 4.6 to 8.9. This pH range represents a change in concentration of nearly twenty thousand fold, in both cases. Nevertheless, there is good evidence that in the carbonate mixtures, where the carbon dioxide concentration is lower and the pH greater, other factors besides the concentration of free carbon dioxide play a part in controlling the rate of photosynthesis. In all probability these factors are the concentrations of bicarbonate and carbonate ions. This opinion is supported by our finding that the rate of photosynthesis in phosphate is remarkably constant from 5 per cent. atmospheric carbon dioxide down to less than 0.05 per cent., while over the same range of concentrations in carbonate mixtures, the rate is known to decline somewhat with declining carbon dioxide concentration.

We cannot say how great a change in internal pH is brought about by changing the external pH from 4.6 to 8.9. That there is some change in internal pH is indicated by the behavior of respiration, which seems to be about 30 per cent. higher at 4.6 than at 7 or 8.9. If we could establish the range of pH at the chloroplast over which photosynthesis is constant, it would help in forming opinions concerning the merits of proposed mechanisms of photosynthesis.

CALIFORNIA INSTITUTE OF TECHNOLOGY  
PASADENA, CALIFORNIA

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# SOIL MOISTURE AS AN INDICATION OF ROOT DISTRIBUTION IN DECIDUOUS ORCHARDS

F. J. VEIHMAYER AND A. H. HENDRICKSON  
(WITH THREE FIGURES)

## Introduction

The study of soil-moisture conditions in an orchard involves, among other things, two perplexing problems that the investigator should clearly understand before the routine work of securing the soil samples is started. One of these is: "What constitutes an adequate sample as regards depth?" And the other: "Where, in the plot, shall the samples be taken?" No two sets of conditions are identical, and the method of sampling used in one case may not give satisfactory results when used in another. It is possible, however, to secure satisfactory results in orchards of mature deciduous trees, provided the soil throughout the area sampled is reasonably uniform in texture, and is of such a nature that it is easily penetrated by roots. Some root stocks, such as the Japanese pear, do not seem to spread through the soil uniformly, and sometimes do not give satisfactory results even when planted on a homogeneous soil.

The experiments reported in this paper give the results of systematic samplings in 13-year-old peach, prune, and walnut orchards growing on a Yolo loam soil at Davis, California. The data show where, with respect to the trunk of the tree, the samples should be taken, and the depth of sampling necessary to secure an adequate record of the use of water by the tree. Inasmuch as the loss of moisture from the soil, below the depth that dries because of surface evaporation, is due, almost entirely, to use of water by the tree, the drying of the soil during the growing season may be used as an indication of the spread and depth of the root system. Data are also given from a 45-year-old pear orchard where the soil-moisture record plainly indicated the lack of uniform distribution of roots.

## Plan of the experiment

During the 1935 season, soil samples were taken in one-foot increments to a depth of 6 feet at monthly intervals in one of the unirrigated plots of the experimental walnut orchard. Four sets of samples of 4 cores each were secured on each sampling date: the first at 8 feet from the trunk of the tree; the second at 12 feet; the third at 16 feet; and the fourth at 20 feet. The first samples were taken on April 24, and the last on October 29, 1935. As the trees were 48 feet apart, the sampling extended almost to the mid-point between the trees. At the time the samples were taken, there was a space of about 10 feet between the ends of branches of adjacent trees. The samples

were placed in tared sheet-metal cans fitted with tight covers, weighed, and then dried in the usual way at 110° C. for forty-eight hours.

In 1936 samples were taken at frequent intervals in another one of the unirrigated plots that entered the growing season with the soil wet to a depth of 12 feet. These samples were taken at three places in each plot, in one-foot increments to a depth of 9 feet, and in a 3-foot increment from 9 to 12 feet. They were taken between 8 and 12 feet from the trunk, the successive points of sampling being moved around the tree in a clockwise direction.

The work of the 1936 season also included an experiment similar to the one carried out in 1935, but was conducted on one of the irrigated plots. The samples were taken in one-foot increments to a depth of 6 feet, at 4, 8, 12, 16, 20, and 24-foot distances from the trunk of the tree. Only two sets of samples were obtained, one on June 12 while there was still readily available moisture remaining from the winter rains, and the second on October 20, while there was still available moisture from the second irrigation. This plot was irrigated on June 15, and again on August 6.

Similar experiments were carried on with peach and prune trees in irrigated plots. Inasmuch as peach and prune trees were planted 24 feet apart, the samples were taken in one-foot increments at 3-, 6-, 9-, and 12-foot spaces from the trunk, and consisted of 4 cores each, taken 90 degrees apart.

### Experimental results

The results obtained in 1935 with walnuts are shown graphically in figure 1. The moisture contents of all samples obtained on April 24 at the beginning of the experiment were approximately at field capacity. The percentage of soil moisture in the top foot decreased uniformly during the first part of the growing season at all sampling distances from the tree. The curves for the individual samples flattened shortly before July 26, and showed a slight loss of moisture in the soil at this depth during August. The slight increase in moisture content late in the season was caused by rain which fell during October and which was held in the top foot.

The second foot likewise showed a fairly uniform decrease in soil moisture until about July 26, after which little, if any, extraction occurred, indicating that the permanent wilting percentage was reached. The results obtained in the third foot were essentially similar to those from the second. The individual samples, however, did not agree as closely as in the second foot, owing, probably to a slight variability in the texture of the soil found at this depth.

The moisture content of the fourth foot was also reduced fairly uniformly at the different sampling distances until July 26. Thereafter, the curves were close together and showed practically no extraction. In the

fifth and sixth feet the extraction of moisture seemed to be slightly more irregular than in the top four feet. At these depths the curves flattened out on August 27, after which extraction of moisture was very slow. It would seem that the readily available soil moisture was not exhausted in the fifth and sixth feet until about a month after it was in the top four feet.

It is interesting to note the comparatively small variation in the results whether the samples were taken 8, 12, 16, or 20 feet from the tree, which indi-

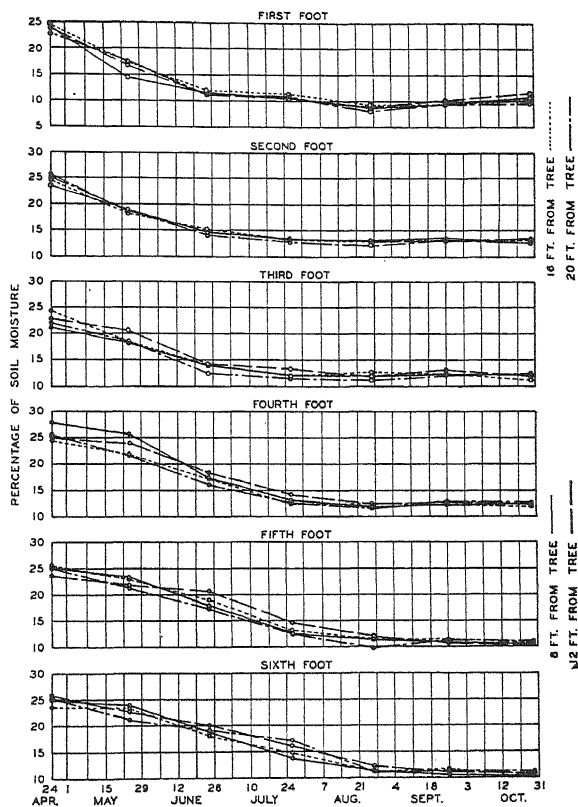


FIG. 1. Moisture conditions in upper six feet of soil in a walnut orchard, Davis, California, in 1935.

cates a fairly complete distribution of roots. The curves obtained from these experiments illustrate the fact that the readily available water at a given depth was extracted about as rapidly near the tree as it was farther away. In other words, the permanent wilting percentage at 20 feet from the trunk of the tree was reached as soon as it was at 8 feet. Furthermore, the permanent wilting percentage was reached in the top four feet about July 26, and about a month later in the fifth and sixth feet.

The results obtained by sampling a walnut orchard at specific distances

from the trunk of the tree in 1936 are given in table I. Each value is the mean of 4 samples taken 90 degrees apart. This plot, which was irrigated twice during the growing season, was sampled on June 12, and again on October 20. Close agreement was obtained at specific depths, regardless of the distance from the tree.

TABLE I

AVERAGE PERCENTAGE OF SOIL MOISTURE IN A WALNUT ORCHARD AT DIFFERENT DEPTHS AND DISTANCES FROM THE TREE, 1936

DISTANCE FROM TREE	DEPTH OF SAMPLE IN FEET					
	0 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Date of sample June 12						
4 feet .....	15.3	17.1	15.1	18.3	22.0	22.4
8 feet .....	14.8	16.4	14.6	18.6	21.4	22.9
12 feet .....	14.6	17.5	14.3	17.5	20.6	24.3
16 feet .....	13.7	16.3	15.6	17.6	20.7	23.5
20 feet .....	13.7	15.7	15.1	15.4	19.0	23.8
24 feet .....	14.0	15.7	15.5	15.6	17.7	23.1
Av.	14.3 ± 0.2	16.4 ± 0.2	15.0 ± 0.3	17.2 ± 0.3	20.2 ± 0.4	23.3 ± 0.5
Date of sample October 20						
4 feet .....	12.9	14.8	13.2	14.8	14.8	14.1
8 feet .....	13.4	14.5	13.6	14.3	15.1	14.4
12 feet .....	12.8	14.9	13.9	13.7	15.0	14.9
16 feet .....	11.7	15.1	13.8	13.5	15.9	15.9
20 feet .....	11.8	14.1	14.2	13.9	14.8	15.2
24 feet .....	10.9	14.2	14.1	13.4	14.8	13.5
Av.	12.2 ± 0.2	14.6 ± 0.2	13.8 ± 0.2	14.0 ± 0.2	15.1 ± 0.3	14.7 ± 0.4

The samples in this plot were taken in each case before the permanent wilting percentage was reached. If the samples were not taken until several days or weeks after the permanent wilting percentage was reached, the results would probably be in agreement, inasmuch as the roots would have had ample time in which to use up the available supply; but in this case the agreement was close even though the samples were taken before the permanent wilting percentage was reached. Furthermore, the results obtained show the uniform use of water at varying distances from the trunk following the wetting of the soil by the even application of water in basins, as well as following the wetting of the soil by the winter rain. Where water is applied in furrows, the irregular distribution of moisture that often results, causes difficulty in interpreting the results secured by soil sampling.

The results obtained in 1936 by frequent sampling to a depth of twelve feet are shown in figure 2. The results obtained with this experiment agree essentially with those obtained in 1935 except that, inasmuch as the work was carried on in another unirrigated plot approximately 500 feet distant from the one used in 1935, the permanent wilting percentages are not the same in each plot. The extraction of moisture in the top foot was fairly

rapid in the early part of the season, but was slower during the latter part of May, and in June, because of light showers and an unusual number of cloudy days. Later in the season the moisture percentage (the sample contained the air-dried, surface soil) was reduced to about 7.5 per cent. The extraction of moisture from the second- and third-foot depths was similar to that from the first foot, the soil moisture being reduced to about the permanent wilting percentage July 15. The soil-moisture reduction in the fourth, fifth, and sixth feet was fairly rapid from the first sampling on April 17 to July 15, when the rate of extraction was materially decreased. After this date the extraction of water was slow. In other words the moisture at this depth was reduced to about the permanent wilting percentage on July 15.

In the seventh-, eighth-, and ninth-foot levels the extraction seemed to be

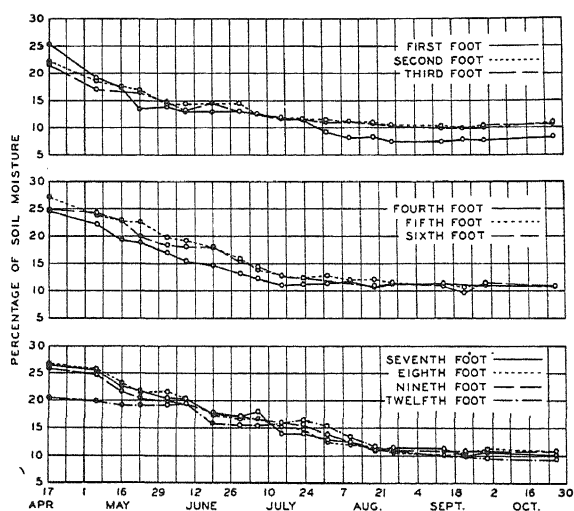


FIG. 2. Soil-moisture conditions in walnut orchard, Davis, California, in 1936.

slightly slower than it did in the top 6 feet of soil. Furthermore, the curves are somewhat more irregular because of variability in the soil. The downward trend of the curves, however, indicates the presence of a fairly dense root population. The permanent wilting percentage was reached August 19 which was about a month after it was reached in the top 6 feet.

The extraction curve for the soil between the 9- and 12-foot levels is somewhat irregular because of the frequency with which coarse textured material was encountered. Nevertheless, it shows that the soil moisture at this depth was reduced from 20.5 per cent. in the middle of April to about 10 per cent. by the middle of September. The reduced rate of extraction after September 14, even though temperatures were high, shows that the permanent wilting percentage was reached on that date.

The results obtained in the peach and prune orchards by sampling in one-foot increments 3, 6, 9 and 12 feet from the tree are given in table II.

TABLE II

AVERAGE PERCENTAGE OF SOIL MOISTURE AT DIFFERENT DEPTHS AND DISTANCES  
FROM THE TREE, JULY 18, 1936

DISTANCE FROM TREE	DEPTH OF SAMPLE IN FEET					
	0 to 1	1 to 2	2 to 3	3 to 4	4 to 5	5 to 6
Peaches						
3 feet .....	10.0	11.5	10.8	11.2	11.7	12.6
6 feet .....	10.8	12.3	11.3	11.6	10.7	11.9
9 feet .....	10.0	11.6	11.2	11.3	11.5	11.6
12 feet .....	13.6	12.3	10.8	9.8	10.7	11.6
Av.	11.1 ± 0.1	11.9 ± .01	11.0 ± 0.1	11.0 ± 0.3	11.2 ± 0.2	11.9 ± 0.2
Prunes						
3 feet .....	10.2	12.7	11.3	10.2	10.8	11.3
6 feet .....	11.0	12.2	10.6	11.1	11.0	10.9
9 feet .....	10.0	11.9	11.1	10.5	10.7	10.5
12 feet .....	9.2 -	11.8	11.4	10.6	10.6	10.2
Av.	10.1 ± 0.2	12.1 ± 0.1	11.1 ± 0.1	10.6 ± 0.2	10.8 ± 0.1	10.7 ± 0.1

The percentages given in the table are the averages of four samples taken from the north, east, south, and west sides of the tree at the distances and depths indicated. The agreement obtained in most cases was very close, as indicated by the small probable errors, and substantiate those from the walnut orchard. These results show what reasonably may be expected when soil-moisture studies are conducted with trees having adequate root distribution and growing on a fairly uniform soil.

On the other hand, while studying the water relations of pear trees in the central coast region of California, the results obtained by soil sampling in an unirrigated plot showed irregularities of considerable magnitude. The trees were on French pear root stocks and were about 45 years old at the time of the experiment. They were planted 20 feet apart on the square system. We assumed that, after 45 years, the soil under the trees was thoroughly permeated by roots at least to the depth ordinarily reached by this species. The soil, which was a Yolo loam, seemed to be uniform to a depth of 6 feet when the preliminary examination was made with the aid of a large post hole auger. As the season progressed, it was evident from the soil-moisture record obtained that either the roots were not uniformly distributed throughout the soil mass, or that layers of soil of different moisture holding properties occurred at varying depths. Careful examination of the samples secured with a soil tube showed considerable differences in texture at specified depths even when taken close to one another.

At the end of the 1934 season a trench, 6 feet deep, was dug about 4 feet



from one of the trees in the dry plot. It was then discovered that at a short distance below the cultivated layer there were distinct layers of soil differing rather widely in texture. In some of these layers there was an abundance of roots of different sizes, while in others, there was a scarcity.

A 12-inch square was laid off on the face of the newly dug trench approximately  $2\frac{1}{2}$  feet below the surface, directly in line with the trunk of the tree, and 36 samples, equidistant from each other, were taken within the 12-inch square by means of a short soil tube. The moisture record was obtained by drying and weighing in the usual manner. The results, expressed as percentages of moisture on the oven dry weight of soil, are given in figure 3. Examination of the results shows the variation in soil moisture that

12"						MEAN
12.7	9.7	14.4	12.8	14.8	15.0	
10.5	11.0	12.2	9.6	11.0	12.0	
10.7	10.8	10.5	12.0	9.8	11.9	
13.1	13.8	15.0	19.7	20.2	11.5	
10.2	21.1	23.2	23.7	24.6	24.1	
21.5	19.3	22.5	23.7	22.6	23.3	
MEAN	13.1	14.3	16.3	16.9	17.2	16.3

FIG. 3. Percentages of soil moisture within a 12-inch square on the face of a trench four feet from the trunk of a pear tree, in the central coast region of California. The samples were taken at the end of the growing season, the top row being  $2\frac{1}{2}$  feet below the surface.

occurred in samples taken a short distance from each other. In one case, a variation in moisture content of 12.5 per cent. occurred with a vertical distance of 2 inches. In general, it will be seen that the moisture contents as shown by the 12 samples taken from the bottom 4 inches on the face of the square with but few exceptions are considerably higher than those taken from the top 6 inches. The six samples taken 8 inches below the top edge of the square, in general, are intermediate between the groups just described. In many cases apparent irregularities in moisture contents may be caused solely by differences in soil texture. In such cases the irregularities may be reduced or eliminated by expressing the moisture content as relative wetness

(ratio between moisture content and moisture equivalent). In this case, plotting the results given in figure 3 on a basis of relative wetness, did not eliminate the variability. The soil under the trees was not of the same degree of wetness even at the end of the growing season. Since this variability was not caused by the texture of the soil, nor by uneven distribution of moisture because the plot received only rainfall, it follows that it was caused by the irregular distribution of roots. It is evident from these data that, although the orchard where these experiments were started was healthy and vigorous, and has been commercially profitable for many years, it was not suitable for experiments that require an accurate interpretation of the soil-moisture conditions existing during the growing season. The presence of layers of different kinds of soil beneath the surface, which prevented the uniform distribution of roots, was obscured by the method of securing the preliminary samples. These were obtained with a post hole auger that tended to pulverize and mix the different layers so that the samples secured appeared fairly homogeneous.

### Discussion and summary

The data presented in this paper show the probable distribution of roots of certain deciduous fruit trees when grown on a reasonably uniform soil. The permanent wilting percentage was reached in a certain depth of soil close to the tree as soon as it was midway between adjacent trees. We have found a few cases, however, in which, because of the kind of root stock or the kind of soil, root distribution was uneven. In these exceptional cases, difficulty was encountered in interpreting the soil-moisture record. Under conditions of soil and root distribution similar to those in this series of experiments, reliance can be placed on a system of soil sampling during the growing season for the planning of a rational irrigation schedule.

Our experience has been that, if a system of soil sampling is to be used in accurately interpreting the plant-soil-moisture relationships during the growing season, the soil upon which the orchard is growing must be fairly uniform. If the soil is variable or if there is an uneven distribution of roots, irregularities in soil moisture of considerable magnitude are frequently found in successive samples. Relative wetness will not aid in interpreting results when root distribution is not uniform. However, when the irregularities in the soil-moisture record are caused by differences in soil texture, they may often be eliminated by using the relative wetness. Under conditions where the irregularities are caused by root distribution, it is better to plan future irrigations on the basis of observations either of the tree itself or of indicator plants growing in the orchard. The chief difficulty in using the response of deciduous trees or indicator plants is the suddenness with which wilting occurs. Ordinarily, the first signs of wilting, as indicated by

a drooping of the leaves, decreased rate of growth of fruit, or other plant responses, occur only a few days before the readily available water is exhausted. If the grower waits for the plants or trees to show visible signs of wilting before preparing for irrigation, the last trees to be irrigated, especially if the area to be watered is large and the irrigating stream is small, sometimes suffer from the lack of water. Regular soil sampling generally gives the grower a much longer period in which to prepare for irrigation, because he can predict with a reasonable degree of accuracy when irrigation will be necessary.

The fact that soil samples taken at any place within the experimental plots in mature peach, prune, and walnut orchards, which have had an even application of water, agree at comparable depths, shows that these trees, under the conditions existing at Davis, have a uniform distribution of roots. Samples taken at any position between the trees may be used to interpret the soil-moisture conditions.

THE UNIVERSITY OF CALIFORNIA

DAVIS, CALIFORNIA



# EFFECT OF SOIL MOISTURE ON THE RATE OF GROWTH OF LONGLEAF AND SLASH PINE SEEDLINGS

L. J. PESSIN  
(WITH ONE FIGURE)

## Introduction

Longleaf pine (*Pinus palustris* Miller) and slash pine (*P. caribaea* Morelet) are widely distributed in the Gulf Coastal Plain. MOHR (12) observed as early as 1896 that under natural conditions longleaf pine thrives on well-drained, sandy soils and that slash pine grows best on moist lands; also, MATTOON (11) found that slash pine is confined mainly to poorly drained lands and borders of swamps and rivers. Similarly PESSIN (13) found that longleaf pine inhabits both dry soils and moist soils but occurs most abundantly on well-drained soils, while slash pine grows principally in moist situations. In plantations, however, slash pine apparently grows as vigorously on the well-drained longleaf-pine lands as on moist soils, and young stands of slash pine under natural conditions sometimes establish themselves on well-drained soils some distance away from their parent trees. These new situations are quite different from those where the parent trees grow. This ability of the slash pine to grow on typical longleaf-pine sites suggests that both of these species may have similar moisture requirements, and that the restricted distribution of slash pine under natural conditions may be due not entirely to moisture conditions but also to other factors. A clear understanding of the moisture requirement of these species of pine is particularly essential for nursery practice, where the ultimate aim is the development of high-grade seedlings to insure high survival and good growth. Such a study as this should also throw light on the choice of suitable sites to plant to each of the species in the large planting program in the South, particularly in Louisiana, Mississippi, and Texas.

This study was undertaken to determine (1) the condition of soil moisture conducive to the best development of longleaf and slash pine seedlings; (2) the maximum and minimum amounts of soil moisture that each of these species can endure; and (3) the amount of dry matter produced by each species under different conditions of soil moisture. This last phase of the problem is essentially a study of the water requirement of these species of pines. By water requirement is meant the ratio of the total amount of water absorbed by the plant during a period of time to the amount of dry matter produced by the plant during that time. The water requirement of a plant is, therefore, a measure of the efficiency with which the water is used. This efficiency can be expressed by the formula  $\frac{L}{D} = W$ , where  $L$  indicates the total

weight of water lost from the plant through transpiration and evaporation;  $D$ , the total weight of the dry matter produced by the plant during the same period of time; and  $W$ , the number of units of water absorbed to produce one unit of dry matter. Inasmuch as the amount of water retained by the plant is relatively insignificant as compared with the amount of water lost, the amount of water transpired by the plant is taken as a measure of the water absorbed.

### Review of literature

The problem of the efficiency of different species in their use of water has interested botanists, agronomists, and foresters for many years. IL'ENKOV (8) as early as 1865 studied the effect of soil moisture on trees. FITTBOGEN (4) found that the production of dry matter in oats was reduced greatly by the reduction of the moisture of the soil. VON HÖHNEL (6, 7) found that there was a definite relationship between the amount of leaf surface and the amount of water consumed by plants.

HELLRIEGEL (5) showed that the water utilization of plants decreased as the soil moisture content was lowered. MAERCKER (10) obtained similar results. VON DASZEWSKI (3) found that the dry weight of tubers was increased greatly in soil with high water content, while KIESSELBACH AND MONTGOMERY (9) found by improved methods that the reduction of the water content from 100 to 60 per cent. produced a reduction of the water requirement. In general, experiments on the effect of the soil moisture content on the water requirement of crop plants showed that the water requirements varied with the water content of the soil.

BRIGGS AND SHANTZ (1) found that field crops differed greatly as regards their efficiency in the use of water. Alfalfa, for example, used four times as much water as did millet to produce one pound of dry matter. In a later paper (2) they reported that even varieties of the same species differed widely in their water requirements.

### Methods

In general, the method used in this work was similar to that used by some of the workers mentioned above. Plants were grown in sealed containers from April to October under greenhouse conditions. The total loss of water and the dry weight of the plants were calculated at the end of the experiment.

### MOISTURE CONDITIONS

Five different moisture conditions were studied. Under "*very dry*" conditions the soil in each container received water amounting to 5 per cent. of the air-dry weight of the soil. Under "*dry*" conditions the water added to the soil amounted to 10 per cent. of the weight of the soil. Under "*damp*" conditions, water amounting to 15 per cent. of the weight of the soil was

added; and under "moist" conditions, 25 per cent. Under the "wet" conditions the soil of the longleaf pine received 35 per cent. of the air-dry weight of the soil, while that of the slash pine received 31 per cent. In both of these wet conditions the soil was saturated, and free water remained standing on the surface.

Inasmuch as it is impossible to maintain a constant moisture content in containers in which plants are growing, no such attempt was made in this study. What was actually studied was the amount of water within certain limits in each series. The uniformity of the distribution of moisture in the containers did not offer a serious problem, since the containers were small and the roots of the trees were well distributed within them.

### SOIL

The soil used in this study belonged to the Ruston fine sandy loam type. It was taken from the surface to a depth of 12 cm. on an area where the vegetation was composed mainly of longleaf pine saplings, species of *Andropogon*, and other piney-woods grasses and herbs. This soil was first passed through a coarse wire screen, then through a fine 2-mm. screen. This screened soil was spread out on canvas and allowed to dry for several days. Samples of the soil were then taken and the moisture content determined on an oven-dry basis. When the moisture content was less than 2 per cent., the soil was considered suitable for use. The soil used had an average moisture content of 1.18 per cent.

A physical analysis of the soil showed the following:

SOIL FRACTION	PER CENT.
Silt .....	26.1
Very fine sand .....	32.0
Fine sand .....	26.0
Sand .....	13.4
Coarse sand .....	2.5
	<hr/>
	100.0

The colloidal content of the soil was 22 per cent. and the initial pH was 5.25.

### CONTAINERS

Two kinds of containers were used. In the very dry, dry, damp, and moist series, paraffined paper milk bottles of 1-quart capacity were used. These were reinforced at the seams with adhesive tape and the entire surface of each was covered with a coat of waterproof shellac. On top of this, a coat of white enamel was applied. The mouth of each bottle was fitted with a paper milk-bottle cap with two holes to permit the passage of the stem of a plant and a glass tube, respectively.

In the wet series, mason jars were used. The mouths of these were closely fitted with paraffined screen-wire discs with two holes like those in the paper caps. Mason jars were used in this series because it was doubtful whether the paper containers could stand the heavy weight of water without becoming weakened and finally leaking.

The advantage in using paper containers was the facility with which the plants could be removed. After the completion of the experiment, the walls of the container were slit open with a sharp knife in several places and the containers placed in a receptacle. A fine stream of water applied to the slits soon loosened all the soil and the roots of the plants were removed without injury or loss of fine rootlets. Removing the roots from the glass jars took considerably longer than from the paper bottles.

#### PREPARATION OF THE CULTURES

Seeds of uniform weight were sown in flats in the same type of soil as that used in the containers. When the cotyledons were completely unfolded, each seedling was weighed and inserted through a hole in the cover of a container, which was then filled with the air-dry soil and weighed. The culture was then watered and allowed to stand until the seedling became well established. At this time the moisture in the culture was brought to the desired content. The lid of the container was then sealed with a mixture of paraffin and beeswax and the culture was again weighed. This final weight constituted the basic weight. Once every week all the cultures were weighed and the loss of water determined. Enough water was then added to bring the cultures back to the original basic weight. Fifty seedlings were used under each condition of soil moisture.

The seedlings were 41 days old at the beginning of the experiment, which lasted from April to the middle of October of the same year. The plants consequently were under observation during their first full growing season.

All the cultures were kept in the greenhouse, where the maximum temperature averaged 96° F. and the minimum 70° F. for the duration of the experiment. The relative humidity averaged 70 per cent. but sometimes reached a minimum of 49 per cent.

#### Analysis of results

The results are shown in table I. A statistical analysis of the data revealed a number of interesting facts. The longleaf pine seedlings showed best development in the moist soil, where the combined dry weight of the tops and roots was highest. The number of fascicles and length of needles were also greatest in the moist soil. Furthermore, in this soil the seedlings absorbed the most moisture, and the rate of transpiration was highest. This was due probably to the vigorous condition of the seedlings. The seedlings



TABLE I  
SUMMARY OF EFFECT OF SOIL MOISTURE ON LONGLEAF AND SLASH PINE SEEDLINGS

SOIL MOISTURE	AVERAGE AMOUNT OF WATER ABSORBED	AVERAGE DAILY TRANSPIRA- TION	AVERAGE NUMBER OF FASCICLES	AVERAGE LENGTH OF		PLANTS WITH MORE THAN 10 LAT- ERAL BRANCHES	PLANTS WITH MORE THAN 10 MYCOR- RHIZA	AVERAGE DRY WEIGHT				WATER RE- QUIRE- MENT	AVER- AGE HEIGHT OF STEM
				cm.	s.c.			gm.	Roots (STEMS PLUS LEAVES)	Roots	TOTAL (TOPS PLUS ROOTS)		
	gm.	s.c.*	no.	cm.	s.c.	%	%	gm.	gm.	gm.	gm.	gm.	cm.
dry (5%)	505.97	53.4908	6.5	30.54	0.8017	61	23	0.359	0.469	1.428	0.1020	354	0.5
(10%)	1,045.49	52.9532	7.3	28.21	0.8102	58	46	1.043	0.669	1.712	0.1005	611	0.5
(15%)	1,384.80	53.4908	8.8	29.06	0.7936	57	51	1.199	0.892	2.091	0.0120	662	0.5
(25%)	2,278.16	52.9532	10.9	34.79	0.7936	82	94	1.902	1.714	3.616	0.1005	630	0.5
(35%)	1,026.54	54.6171	8.9	28.61	0.8102	40	49	1.259	0.771	2.030	0.1039	506	0.5
dry (5%)	373.82	57.1009	7.4	9.10	0.5892	63	51	0.472	0.554	1.026	0.1086	364	8
(10%)	1,172.83	53.4908	14.9	13.29	0.4810	82	73	1.011	1.270	2.281	0.1020	514	12
p (15%)	1,666.39	53.4908	16.4	14.81	0.4707	82	84	1.165	1.185	2.350	0.1020	709	12
t (25%)	2,115.39	53.4908	17.3	15.18	0.4610	86	96	1.331	1.081	2.412	0.1020	877	14
(35%)	1,038.66	56.4483	9.3	11.31	0.5167	29	43	0.527	0.366	0.893	0.1072	1,163	10

[ error.

in this moist soil had the largest number of lateral roots and the largest number of mycorrhiza. The taproots were shorter than in the wet cultures but longer than in the other cultures.

The poorest development occurred in the very dry soil. Here the longleaf pine seedlings had the lowest dry weight, the fewest fascicles, the shortest taproots, and the fewest mycorrhiza. The seedlings in this soil had the lowest transpiration rate, and absorbed the least water. The needles were shorter than those in the moist soil but did not differ significantly from those growing in the other soils. The lateral roots were fewer than in the moist soil but more than in the wet soil.

The longleaf pine seedlings growing in the wet soil absorbed more water than did those growing in the very dry soil, about the same amount as those growing in the dry soil, and considerably less than those in the other two cultures. In this wet soil the seedlings transpired less than in the moist soil but more than those in the other cultures. The difference between the rate of transpiration in the seedlings in this soil and that of the seedlings in the damp soil was not very significant, and may be due to the fact that the two had the same number of fascicles. The seedlings in the wet soil had shorter needles than those in the very dry and in the moist soils. The taproots of the seedlings in this wet soil were longer than those in any of the other cultures, but the lateral roots were fewest. There were half as many mycorrhiza in the wet soil as in the moist, but twice as many as in the very dry soil. The dry weight of the seedlings in the wet soil was less than that of the seedlings in the moist soil, but higher than the dry weight of the seedlings in the very dry and dry soils.

The slash pine seedlings also absorbed the most water in the moist soil and had the highest transpiration rate in this soil. Also, the fascicles were more numerous and the needles were longer in the moist soil than in the other cultures. The taproots were shortest in the very dry and wet soils and longest in the damp and moist soils. The lateral and mycorrhizal roots were most numerous in the moist soil. The dry weight of the seedlings was highest in the moist soil, but did not differ significantly from that in the dry and damp soils. If the quality of the seedlings were to be judged by their dry weight, then the slash pine, unlike the longleaf pine, had no pronounced optimum condition, so that the seedlings appeared of about the same quality in the dry, damp, and moist soil, but were distinctly better than those in the extreme very dry and wet conditions.

In the wet soil the seedlings absorbed three times as much water as those growing in the very dry soil, but significantly less than those growing in the damp and moist soils. The transpiration rate in the wet soil was also three times higher than in the very dry soil, but considerably lower than in the damp and moist soil. The fascicles were slightly more numerous and the

needles were longer in the wet soil than in the very dry soil. The length of the taproot was about the same in both the wet and the very dry soils, but the number of lateral roots and mycorrhizal branches was considerably lower in the wet than in the very dry soil.

### Discussion

The results (table I, fig. 1) indicate that both longleaf and slash pine seedlings showed best development in the soil which had an abundance of moisture but which was not completely saturated. In this moist soil the seedlings had a high transpiration rate and absorbed much water, with the result that here occurred the best development of the seedlings. Of interest also is the fact that the rate of transpiration was nearly the same for slash as for longleaf pine seedlings of the same age and in soils of similar moisture content. There also apparently exists a close relationship between the rate of transpiration and the moisture content of the soil; the transpiration rate was lowest in the very dry soil and highest in the moist soil, but in the wet soil it was low again.

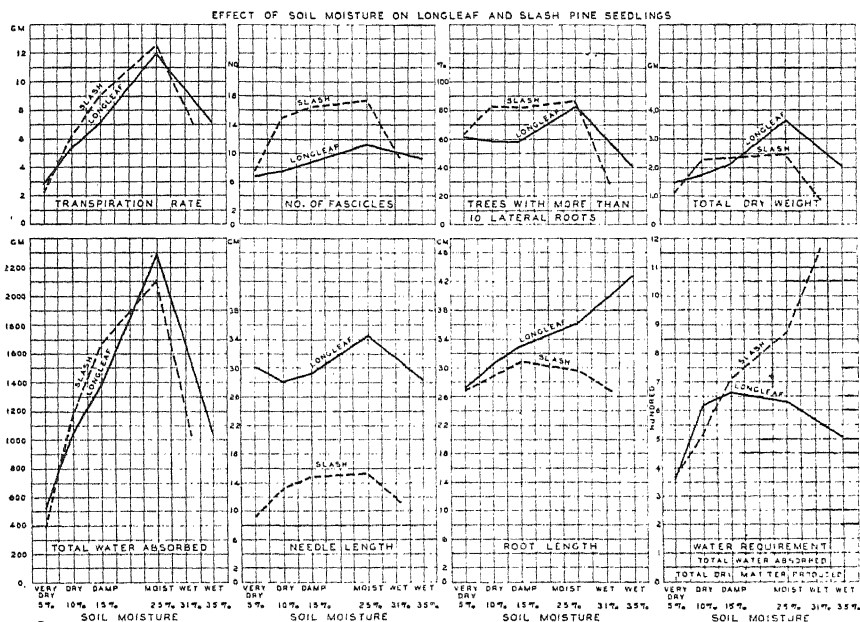


FIG. 1. Effect of soil moisture on longleaf and slash pine seedlings.

The commonly expressed idea that longleaf pine is a dry-land plant and that slash pine prefers wet sites is supported by the water requirement of the two species. In the early seedling stage, however, both species apparently

can endure extreme dry and extreme wet conditions. The mortality of slash pine in the very dry soil was higher than that of the longleaf in the same soil, and the mortality of longleaf was higher in the wet soil than that of the slash pine, but most of the seedlings of each species survived under the extreme conditions. The optimum moisture condition for growth is not markedly different for both species, but the slash pine seedlings apparently have a wider range of optimum moisture conditions than longleaf pine. The fact that under natural conditions longleaf pine is more abundant on dry sites and slash pine on wet sites may be caused by frequent fires which often do not seriously injure the longleaf pine seedlings, but sometimes annihilate whole stands of slash pine seedlings. The absence of longleaf pine seedlings on wet sites may be due not entirely to the wetness of the soil but to the dense vegetation shading out the longleaf pines and also to the fact that longleaf takes so long to start height growth. Slash pine seedlings apparently can endure considerably more shade than can longleaf pine seedlings.

Optimum moisture conditions are necessary for good development of the foliage of both longleaf and slash pine seedlings. Slash pine seedlings produced more fascicles than did the longleaf seedlings under all conditions, but the length of needles of the seedlings growing under optimum conditions (moist soil) was greater for the longleaf than for the slash pine.

The effect of soil moisture on root development showed some differences between the two species. In the wet soil the roots of the longleaf were considerably longer than those of the slash pine, but in the dry soil there was practically no difference. In the moist soil, however, the longleaf pine seedlings produced longer roots than those of the slash pine. The roots of the longleaf seedlings in the wet soil were over 50 per cent. longer than those in the very dry soil, while the slash pine roots in the wet soil and those in the very dry soil were about equal in length.

Of particular interest is the fact that in both longleaf and slash pine seedlings the roots of the most vigorous seedlings possessed the most mycorrhiza. The close relationship between the presence of mycorrhiza and the vigor of the pine seedlings is noteworthy and deserves further study.

The efficiency with which each of the pine seedlings utilizes the water is shown by their respective water requirements. For the sake of comparison, the water requirement of the seedlings in the very dry soil was expressed as 1 for both slash and longleaf pine. The ratios are shown in table II. It is evident that the efficiency of the pine seedlings in their utilization of water to produce dry matter differed not only with the moisture content of the soil but also with the species. In the very dry soil, both species were nearly alike in their water requirement, but with the increase in the moisture content the slash pine seedlings became less efficient; while the longleaf pine

TABLE II

RATIOS OF WATER REQUIREMENTS FOR LONGLEAF AND SLASH PINES IN SOILS OF VARYING MOISTURE CONTENT

SPECIES	VERY DRY	DRY	DAMP	MOIST	WET
Longleaf .....	1	1.7	1.9	1.8	1.4
Slash .....	1	1.4	1.9	2.4	3.1

seedlings showed no marked difference except in the wet soil, where the ratio was somewhat lower than in the other cultures.

A significant fact obtained in this study is that both longleaf and slash pine seedlings during their first year of growth apparently can endure very dry and wet soils. The longleaf pine can tolerate the dry conditions a little better than the slash pine seedlings, and the latter apparently can endure extreme wetness somewhat better than the longleaf; but the best conditions for growth apparently lie within the same limits of soil moisture for both species, the slash pine having a little wider range of favorable moisture condition. A soil that drains too rapidly or one that drains poorly apparently is not conducive to good growth and development of either species. In the light of this fact, it is possible to understand why slash pines growing on soils with underlying hardpan where the drainage is poor generally exhibit a stunted condition. Similarly, the absence of longleaf pine seedlings in savannas may also be caused by a condition of poor drainage. The savannas, which in many cases are old cut-over longleaf pine lands, possessed, before the removal of the trees, an upward drainage through the transpiration of the mature trees, but when these were cut, the soil (naturally poorly drained) became saturated owing to reduction of this upward drainage, making the site unfavorable for the establishment and growth of the longleaf pine seedlings.

The results of this study are particularly adaptable to nursery practice. If a watering schedule can be arranged in the nursery by which the optimum soil moisture can be maintained, a good grade of pine seedlings can be developed, thereby eliminating waste in culling.

### Summary

1. Longleaf and slash pine seedlings were grown in cultures under different conditions of moisture including very dry, dry, damp, moist, and wet soils. In these the original total amount of moisture was brought back by frequent replacement of the moisture lost through transpiration.

2. The results indicate that longleaf pine seedlings during their first growing season exhibit the best growth in moist soils where the initial moisture amounts to about 25 per cent. of the total weight of the soil.

3. Slash pine is apparently not as exacting as longleaf pine in its moisture needs. No appreciable differences in growth are exhibited in cultures where the initial moisture ranges from 15 to 25 per cent. of the total weight of the soil. While both longleaf and slash pine seedlings, showed a high survival under extreme conditions of dryness and wetness, under these conditions they produced poorest growth.

4. Under moist conditions many mycorrhiza were produced, and all seedlings bearing mycorrhiza exhibited a particularly vigorous appearance.

5. The transpiration rates for both longleaf and slash pine seedlings were very similar; in both the rate varied with the amounts of moisture in the soil.

6. The water requirement generally was higher for slash pine under moist to wet conditions than for the longleaf pine seedlings under the same moisture conditions. The results indicate that slash pine seedlings require more moisture to build a unit of dry matter than do the longleaf pine seedlings.

SOUTHERN FOREST EXPERIMENT STATION  
NEW ORLEANS, LOUISIANA

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# TIME FACTOR CONCERNED WITH FLUIDITY CHANGES IN WARMED AND COOLED CELLS OF *ZYGNEMA* AND *SPIROGYRA*<sup>1</sup>

HENRY T. NORTHEN  
(WITH THREE FIGURES)

## Introduction

It is generally known that the viscosity of hydrophilic colloids changes during a long period after temperature equilibrium has been reached. BÊLEHRÁDEK (1) assumes that protoplasm responds to low temperatures as do hydrophilic colloids *in vitro*, and he suggests that the gradual slowing up of vital processes when cells are cooled to biological zero may be the result of a gradual increase in the viscosity of protoplasm. In other words, BÊLEHRÁDEK believes that the rate of diffusion, which in turn is dependent upon protoplasmic viscosity, may be slower than the rate of chemical change as such; and, hence, in a catenary series of reactions the rate may be limited by protoplasmic viscosity.

The data presented in this paper, however, indicate that when cells are warmed or cooled the viscosity of the protoplasm becomes constant when the temperature equilibrium is reached. Hence the fact that the rate of biological processes in cooled cells does not become constant when the temperature equilibrium is reached cannot be used as an argument in favor of the hypothesis that diffusion may limit the rate of a catenary series of reactions.

## Materials and methods

For the determination of the time-factor when cells are cooled from 20° C. to 1° C., filaments of *Zygnema* and *Spirogyra* from water at 20° C. were laid between two strips of cotton which had been soaked in water at 20° C. The cotton wads containing the filaments were inserted in centrifuge tubes immersed in water maintained at 1° C. After the filaments had been in the ice water the desired time (tables II and V), they were centrifuged, still in ice water, with a hand-driven centrifuge. The filaments were then fixed and mounted in a 1 per cent. chromo-acetic solution and the distances the chloroplasts had been moved were determined. For *Zygnema*, the distances the chloroplasts had been moved were determined as previously described by NORTHEN (2). For *Spirogyra* no measurable criterion could be used, but the comparative displacements of the chloroplasts has been recorded by means of plus signs.

For the determination of the time-factor when cells are warmed from

<sup>1</sup> Contributions from the Department of Botany and the Rocky Mountain Herbarium of the University of Wyoming, no. 163.

TABLE I  
MINUTES OF EXPOSURE NECESSARY FOR THE FLUIDITY (DISTANCE CHLOROPLASTS MOVED) TO BECOME CONSTANT WHEN CELLS  
OF *Zygnema* WERE WARMED TO 20° C.

EXPERIMENT NUMBER	NUMBER OF CELLS MEASURED	AVERAGE LENGTH OF CELLS	FORCE X GRAVITY	SECONDS CENTRIFUGED	MINUTES IN WATER AT 20° C.	DISTANCE CHLOROPLASTS MOVED	$\sigma$	CELLS COLLECTED DURING
1 .....	$\left\{ \begin{array}{l} 44 \\ 48 \\ 42 \\ 40 \end{array} \right\}$	$\left\{ \begin{array}{l} 3.3 \\ 3.25 \\ 3.2 \\ 3.12 \end{array} \right\}$	$\left\{ \begin{array}{l} \\ \\ \\ 170 \end{array} \right\}$	$\left\{ \begin{array}{l} \\ \\ 90 \\ \end{array} \right\}$	$\left\{ \begin{array}{l} 0.0 \\ 0.5 \\ 1.5 \\ 5.0 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.41 \pm 0.024 \\ 0.941 \pm 0.02 \\ 1.03 \pm 0.014 \\ 1.02 \pm 0.01 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.234 \\ 0.202 \\ 0.134 \\ 0.126 \end{array} \right\}$	$\left\{ \begin{array}{l} \text{September} \\ " \\ " \\ " \end{array} \right\}$

1° C. to 20° C., filaments of *Zygnema* and *Spirogyra* were laid between two strips of cotton which had been soaked in water at 20° C. The cotton wads were inserted in the centrifuge tubes in water at 1° C. After the filaments had been in the ice water for ten minutes they were transferred to water at 20° C. After the filaments had been in the water the desired time (tables I and IV) they were centrifuged, mounted, fixed, and the displacements of the chloroplasts were determined as previously described.

To determine the approximate time necessary for a temperature equilibrium to be reached, a thermometer was laid between two strips of cotton soaked in water at 20° C. The cotton wad containing the bulb of the thermometer was then inserted in a centrifuge tube immersed in water at 1° C. The temperature was read at intervals of thirty seconds. A similar method was used to determine the time necessary for equilibrium to be reached when the temperature was raised from 1° C. to 20° C.

### Data and conclusions

The approximate time necessary for temperature equilibrium to be established in the cotton wads is expressed graphically in figure 1. The results of the experimentation on *Zygnema* are recorded in tables I and II and in figure 2.

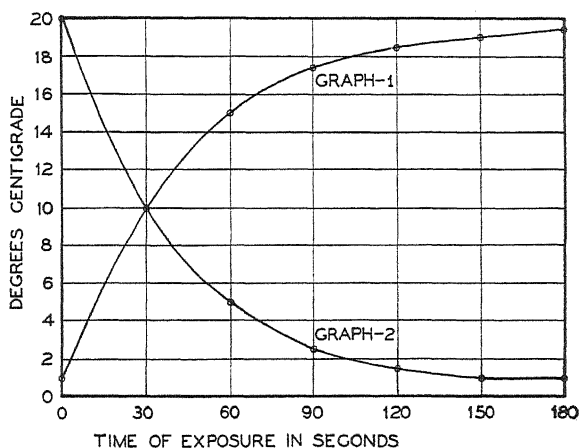


FIG. 1. Seconds exposure necessary for equilibrium to be established in cotton wads which were immersed in water at 20° C. (graph 1) or at 1° C. (graph 2).

In the preceding tables "length of cells" and "distance moved" are expressed in relative units—the units on the ocular micrometer which was used in measuring the displacements. The values may be converted into microns by multiplying by 34.75.

It will be observed, graph 1 of figure 2, that when cells are transferred from water at 1° C. to water at 20° C. the fluidity increases and becomes

**TABLE II**  
**MINUTES OF EXPOSURE NECESSARY FOR THE FLUIDITY (DISTANCE CHLOROPLASTS MOVED) TO BECOME CONSTANT WHEN CELLS OF**  
*Zygnuma* **WERE COOLED TO 1° C.**

EXPERIMENT	NUMBER OF CELLS MEASURED	AVERAGE LENGTH OF CELLS	FORCE X GRAVITY	SECONDS CENTRIFUGAL	MINUTES IN WATER AT 1° C.	DISTANCE CHLOROPLASTS MOVED	$\sigma$	CELLS COLLECTED DURING
2	$\begin{Bmatrix} 54 \\ 47 \\ 60 \end{Bmatrix}$	$\begin{Bmatrix} 3.35 \\ 3.27 \\ 3.13 \end{Bmatrix}$	$\left. \begin{array}{c} \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \end{array} \right\} 170$	$\left. \begin{array}{c} \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \end{array} \right\} 90$	3	$0.701 \pm 0.026$	0.285	September
					6	$0.609 \pm 0.029$	0.293	
					12	$0.604 \pm 0.017$	0.205	
					0	$1.139 \pm 0.026$	0.265	
					3	$0.71 \pm 0.014$	0.146	
					6	$0.65 \pm 0.028$	0.301	
3	$\begin{Bmatrix} 48 \\ 48 \\ 54 \\ 50 \\ 51 \\ 77 \end{Bmatrix}$	$\begin{Bmatrix} 3.35 \\ 3.5 \\ 3.45 \\ 3.20 \\ 3.22 \\ 3.21 \end{Bmatrix}$		$\left. \begin{array}{c} \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \end{array} \right\} 35$	12	$0.624 \pm 0.024$	0.253	" "
					24	$0.625 \pm 0.017$	0.182	
					48	$0.639 \pm 0.017$	0.218	
					0	$0.275 \pm 0.008$	0.097	
					2	$0.10 \pm 0.006$	0.06	
					6	$0.075 \pm 0.004$	0.065	
4	$\begin{Bmatrix} 60 \\ 52 \\ 50 \\ 50 \end{Bmatrix}$	$\begin{Bmatrix} 2.00 \\ 1.99 \\ 2.08 \\ 1.97 \end{Bmatrix}$		$\left. \begin{array}{c} \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \end{array} \right\} 60$	12	$0.133 \pm 0.007$	0.078	October
					2	$0.08 \pm 0.006$	0.059	
					12	$0.08 \pm 0.003$	0.029	
					0	$0.385 \pm 0.009$	0.08	
					3	$0.14 \pm 0.006$	0.06	
					6	$0.19 \pm 0.008$	0.086	
5	$\begin{Bmatrix} 49 \\ 52 \\ 50 \\ 44 \end{Bmatrix}$	$\begin{Bmatrix} 2.05 \\ 1.92 \\ 1.92 \\ 1.84 \end{Bmatrix}$		$\left. \begin{array}{c} \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \end{array} \right\} 50$	9	$0.177 \pm 0.004$	0.047	" "
					0	$0.272 \pm 0.008$	0.097	
					1	$0.244 \pm 0.006$	0.076	
					3	$0.161 \pm 0.005$	0.073	
					6	$0.166 \pm 0.006$	0.073	
					0	$0.44 \pm 0.009$	0.079	
6	$\begin{Bmatrix} 71 \\ 63 \\ 60 \\ 70 \end{Bmatrix}$	$\begin{Bmatrix} 2.03 \\ 2.13 \\ 2.10 \\ 1.97 \end{Bmatrix}$		$\left. \begin{array}{c} \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \end{array} \right\} 382$	1	$0.300 \pm 0.01$	0.093	" "
					2	$0.171 \pm 0.01$	0.099	
					4	$0.169 \pm 0.009$	0.069	
					0	$0.238 \pm 0.005$	0.051	
					1	$0.122 \pm 0.003$	0.058	
					2	$0.09 \pm 0.004$	0.048	
7	$\begin{Bmatrix} 38 \\ 42 \\ 38 \\ 29 \end{Bmatrix}$	$\begin{Bmatrix} 2.11 \\ 2.08 \\ 2.03 \\ 2.10 \end{Bmatrix}$		$\left. \begin{array}{c} \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \\ \text{ } \end{array} \right\} 12$	4	$0.072 \pm 0.004$	0.044	" "
					0			
					1			
					2			
					4			
					0			
8	$\begin{Bmatrix} 41 \\ 57 \\ 54 \\ 50 \end{Bmatrix}$	$\begin{Bmatrix} 1.42 \\ 1.43 \\ 1.40 \\ 1.44 \end{Bmatrix}$			1			
					2			
					4			
					0			
					1			
					2			
					4			
9	$\begin{Bmatrix} 41 \\ 57 \\ 54 \\ 50 \end{Bmatrix}$	$\begin{Bmatrix} 1.42 \\ 1.43 \\ 1.40 \\ 1.44 \end{Bmatrix}$			1			
					2			
					4			
					0			
					1			
					2			
					4			

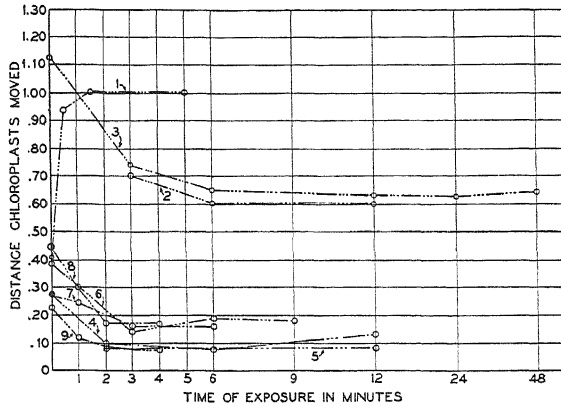


FIG. 2. Minutes exposure necessary for the fluidity (distance chloroplasts moved) to become constant when cells of *Zygnema* were warmed to 20° C. (graph 1) or cooled to 1° C. (graphs 2-9).

relatively constant within half a minute. An examination of graph 1 of figure 1 indicates that at the end of thirty seconds the probable temperature of the cells is only ten degrees. This indicates that the decided increase in fluidity occurs between 0° and 10° C. and that above 10° C. the increase in fluidity is much less marked. Moreover, the data indicate that when cells are warmed the fluidity becomes constant as rapidly as the temperature equilibrium is established.

Graphs 2 and 3 of figure 2, for long cells (hence, a greater distance to move), seems to indicate that the time necessary for the fluidity of the cytoplasm to become constant is more than three minutes and less than six minutes. However, graphs 4 to 9 (for shorter cells) indicate that the time

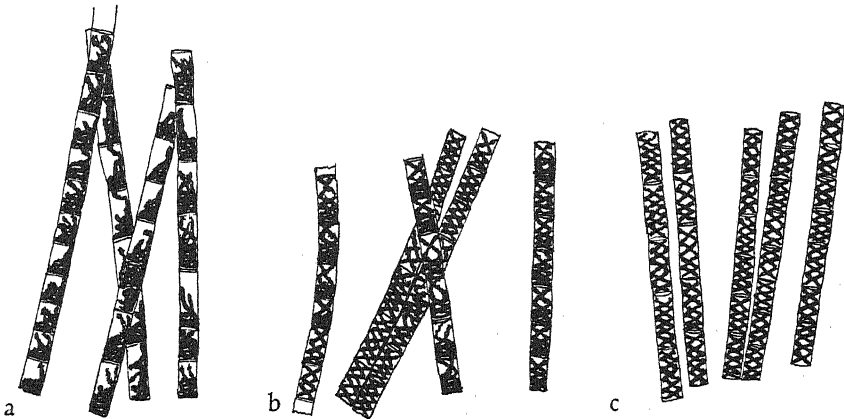


FIG. 3. Cells centrifuged for 45 seconds with a force of  $680 \times$  gravity. a, maintained in water at 20° C.; b, maintained in water at 1° C. for one minute; c, maintained in water at 1° C. for two minutes.

necessary for the fluidity to become constant is less than two minutes and more than one minute. In graphs 2 and 3 the higher fluidity at three minutes as contrasted with the fluidity at six or more minutes is probably spurious and may have resulted from a failure to stir vigorously enough the mixture of ice and water in which the filaments were immersed.

Graph 2 of figure 1 indicates that about two minutes are required for the temperature equilibrium to be established, and graphs 4 to 9 of figure 2 indicate that two minutes are required for the fluidity to reach a minimum. Hence, it becomes reasonably certain that as the temperature equilibrium is reached the fluidity becomes constant. In other words, the time factor for the fluidity to become constant when cells of *Zygynema* are warmed from 1° to 20° C. or cooled from 20° to 1° C. is zero.

The question next arises as whether or not phases more viscous than cytoplasm have a time factor of zero. In *Spirogyra* at least two factors determine the ease of displacing the chloroplast. First, the viscosity of the hyaline cytoplasm, and second, the ease with which the more viscous chloroplast can be bent. It is probable that the second factor is the limiting one, and hence, in *Spirogyra*, studies of displacements of chloroplasts give the time factor for the consistency of the more viscous chloroplast to become constant.

The results of the experimentation on *Spirogyra* are recorded in tables III, IV, and V and in figure 3.

TABLE III

RELATIVE DISPLACEMENTS OF THE CHLOROPLASTS AFTER TEN MINUTES EXPOSURE TO WATER  
AT TEMPERATURES OF 0°, 5°, 10°, 15°, AND 20° C.

TEMPERATURE	FORCE × GRAVITY	SECONDS CENTRIFUGED	RELATIVE DISPLACEMENT
0° C.	} 680	} 45	trace
5			++
10			++++
15			+++++
20			+++++

TABLE IV

RELATIVE DISPLACEMENTS OF CHLOROPLASTS IN CELLS OF *Spirogyra*  
MAINTAINED AT 1° C. FOR 10 MINUTES  
AND THEN MAINTAINED AT 20° C. FOR DIFFERENT PERIODS OF TIME

SECONDS OF EXPOSURE AT 20° C.	FORCE × GRAVITY	SECONDS CENTRIFUGED	RELATIVE DISPLACEMENT
0	} 680	} 45	trace
30			+++
60			+++++
90			+++++
120			+++++

TABLE V

RELATIVE DISPLACEMENTS OF CHLOROPLASTS IN CELLS OF *Spirogyra* MAINTAINED AT 20° C. THEN MAINTAINED AT 1° C. FOR DIFFERENT PERIODS OF TIME

SECONDS OF EXPOSURE AT 1° C.	FORCE × GRAVITY	SECONDS CENTRIFUGED	RELATIVE DISPLACEMENT
0	} 680	} 45	+++++
60			++
90			+
120			trace
150			trace
180			trace

The experiments recorded in tables III, IV, and V are representative ones. Each experiment has been repeated six times by the author or others in this laboratory and each time the same results have been obtained.

Graph 1 of figure 1 indicates that the probable temperature of the filaments after 60 seconds exposure was 15° C. It will be noted (table IV) that the displacement following sixty seconds exposure to water at 20° is the same as the displacement recorded in table III for material maintained at 15° C. At the end of ninety seconds the probable temperature of the filaments was 17.5° C. Upon comparing the value in table IV after ninety seconds exposure with the value at 15° or 20° C. in table III, it will be noted that the values are identical. Hence, in warmed cells of *Spirogyra* as well as in warmed cells of *Zygnema* the fluidity becomes constant as rapidly as the temperature equilibrium is reached.

If comparisons are made between graph 2 of figure 1 and tables III and V, it will be noted that when cells of *Spirogyra* are cooled from 20° C. to 1° C. the fluidity of the chloroplast becomes constant as rapidly as the temperature equilibrium is reached.

The experiments on *Zygnema* and *Spirogyra* demonstrate that when cells are warmed or cooled the consistency of the cytoplasm and the more viscous chloroplast becomes constant as rapidly as the temperature equilibrium is established. The time factor is zero, and hence it is doubtful that the gradual slowing up of vital processes when cells are cooled can be explained on the basis of a gradual increase in protoplasmic viscosity.

### Summary

1. Filaments of *Zygnema* sp. and *Spirogyra* sp. were transferred from water at 20° C. to water at 1° C., centrifuged after various periods of time, and the distances the chloroplasts had moved were determined. Other filaments of *Zygnema* and *Spirogyra*, which were maintained at 1° C. for ten minutes, were transferred to water at 20° C., and after various time intervals were likewise centrifuged, and the displacements determined.

necessary for the fluidity to become constant is less than two minutes and more than one minute. In graphs 2 and 3 the higher fluidity at three minutes as contrasted with the fluidity at six or more minutes is probably spurious and may have resulted from a failure to stir vigorously enough the mixture of ice and water in which the filaments were immersed.

Graph 2 of figure 1 indicates that about two minutes are required for the temperature equilibrium to be established, and graphs 4 to 9 of figure 2 indicate that two minutes are required for the fluidity to reach a minimum. Hence, it becomes reasonably certain that as the temperature equilibrium is reached the fluidity becomes constant. In other words, the time factor for the fluidity to become constant when cells of *Zygynema* are warmed from 1° to 20° C. or cooled from 20° to 1° C. is zero.

The question next arises as whether or not phases more viscous than cytoplasm have a time factor of zero. In *Spirogyra* at least two factors determine the ease of displacing the chloroplast. First, the viscosity of the hyaline cytoplasm, and second, the ease with which the more viscous chloroplast can be bent. It is probable that the second factor is the limiting one, and hence, in *Spirogyra*, studies of displacements of chloroplasts give the time factor for the consistency of the more viscous chloroplast to become constant.

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0° C.	} 680	} 45	trace
5			++
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15			+++++
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TABLE IV

RELATIVE DISPLACEMENTS OF CHLOROPLASTS IN CELLS OF *Spirogyra*  
MAINTAINED AT 1° C. FOR 10 MINUTES  
AND THEN MAINTAINED AT 20° C. FOR DIFFERENT PERIODS OF TIME

SECONDS OF EXPOSURE AT 20° C.	FORCE × GRAVITY	SECONDS CENTRIFUGED	RELATIVE DISPLACEMENT
0	} 680	} 45	trace
30			+++
60			+++++
90			+++++
120			+++++



TABLE V

RELATIVE DISPLACEMENTS OF CHLOROPLASTS IN CELLS OF *Spirogyra* MAINTAINED AT 20° C. THEN MAINTAINED AT 1° C. FOR DIFFERENT PERIODS OF TIME

SECONDS OF EXPOSURE AT 1° C.	FORCE × GRAVITY	SECONDS CENTRIFUGED	RELATIVE DISPLACEMENT
0	} 680	} 45	+++++
60			++
90			+
120			trace
150			trace
180			trace

The experiments recorded in tables III, IV, and V are representative ones. Each experiment has been repeated six times by the author or others in this laboratory and each time the same results have been obtained.

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The experiments on *Zygnema* and *Spirogyra* demonstrate that when cells are warmed or cooled the consistency of the cytoplasm and the more viscous chloroplast becomes constant as rapidly as the temperature equilibrium is established. The time factor is zero, and hence it is doubtful that the gradual slowing up of vital processes when cells are cooled can be explained on the basis of a gradual increase in protoplasmic viscosity.

### Summary

1. Filaments of *Zygnema* sp. and *Spirogyra* sp. were transferred from water at 20° C. to water at 1° C., centrifuged after various periods of time, and the distances the chloroplasts had moved were determined. Other filaments of *Zygnema* and *Spirogyra*, which were maintained at 1° C. for ten minutes, were transferred to water at 20° C., and after various time intervals were likewise centrifuged, and the displacements determined.

2. The results demonstrate that when cells are warmed to 20° C. or cooled to 1° C. the consistency of the protoplasm becomes constant as rapidly as the temperature equilibrium is established. The time factor is zero.

The writer takes pleasure in expressing his gratitude to Mr. MERRITT BODDY and to Mr. MICHAEL BARA of this department for aid in measuring displacements within cells.

UNIVERSITY OF WYOMING,  
LARAMIE, WYOMING

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# META- AND PYROPHOSPHATE WITHIN THE ALGAL CELL

ANNA L. SOMMER AND THOMAS E. BOOTH

## Introduction

Comparatively little attention has been given to the pyro and almost none to the meta form of phosphate in plant and animal studies. Since reporting the occurrence of pyrophosphate in muscle in 1928, LOHMANN (5, 6, 7, 8, 9, 10, 11, 12) has conducted a number of experiments on the occurrence and transformation of pyrophosphate in animal tissue. He also made a few similar studies with yeast and plant material. Other work in which the pyro form of phosphate was considered was done by MEYERHOF and LOHMANN (14), BOYLAND (1), LEVITOV (4), EGGLETON and EGGLETON (2), EMBDEN, HEFTER, and LEHNARTZ (3), NEEDHAM and VAN HEYNINGEN (15), and ROCHE (16). According to LOHMANN (7) and BOYLAND (1), about one-fourth of the phosphate in yeast is in the pyro form. LEWITOW (4) reported that incubation of brewer's yeast did not alter the pyrophosphate content but that when this was done in the presence of glucose there was a considerable loss in pyrophosphate with a simultaneous decrease in orthophosphate. In most of the work, determinations for pyrophosphate might have included metaphosphate. WEISSFLOGG and MENGDEHL (19) found meta- and pyrophosphate in the roots of corn only when they used these forms as the source of phosphate in their culture media. Even in these cases they were unable to detect either meta- or pyrophosphate in the upper parts of the plant and concluded that these were converted into the ortho form before they reached the stems and leaves. Dr. RALPH MORGAN (private conversation) said that he believed metaphosphates were an important form in the living organism because they readily form complexes. An investigation was therefore undertaken to determine whether metaphosphate was present in living green plants and, if so, its relation to the presence of the ortho and pyro forms.

## Experimentation

The ease with which meta- and pyrophosphates are converted to the ortho form makes it desirable to use, if possible, the intact living organism. The senior author and colleagues (17, 18) have shown that the ALLISON magneto-optic apparatus permits the direct detection of compounds within sufficiently small and transparent plants. The only requirement is that sufficient light be transmitted through the organism to give the minimum or minima of the compound being studied.

A pure culture of *Chlorella pyrenoidosa*<sup>1</sup> was used in most of the studies but similar results were obtained with other algae.

<sup>1</sup> The culture was obtained from Dr. D. APPLEMAN of the University of California at Los Angeles and was of a strain isolated by Dr. R. EMERSON of the California Institute of Technology.

Preliminary trials were made to determine if metaphosphate was present within the algal cell. A suspension of algae in a nutrient solution in which all of the necessary ions were present was placed in an observation tube and the minima for  $KPO_3$  were sought. These were found to be present and angle readings<sup>2</sup> to within  $\pm 0.5^\circ$  were made. The suspensions were filtered through hardened filter paper, and readings were made on the filtrate. The filtrate usually showed no metaphosphate; and when the minima for this compound could be seen, the angle readings were very small.

After the preliminary tests showed the presence of metaphosphate within the algal cell, a study was made of its formation from the orthophosphate in the solution.

A culture solution was made up as follows:

MgSO <sub>4</sub> .....	10	p.p.m.
CaSO <sub>4</sub> .....	10	p.p.m.
KCl .....	12.5	p.p.m.
KNO <sub>3</sub> .....	10	p.p.m. to 100 p.p.m.

A small amount of a heavy algal suspension from a culture in which all or nearly all of the phosphate had been utilized was added to this solution. Potassium acid phosphate was added to the suspension just before making the readings. Readings for  $K_3PO_4$ <sup>3</sup> and  $KPO_3$  were made immediately after

<sup>2</sup> Angle readings vary directly with the concentration. BISHOP, E. R., DOLLINS, C. B., and OTTO, I. G. Magneto-optic rotation method for quantitative determination of calcium. Jour. Amer. Chem. Soc. 55: 4365-4370. 1933.

The data presented in table II were obtained by the senior author during the absence of the junior author. The readings, although much higher than those in table I, which the junior author observed, represent lower concentrations. Readings obtained by different observers may vary greatly for a given concentration as is shown in the following data.

COMPARISON OF ANGLE READINGS FOR THE SAME CONCENTRATION BY TWO OBSERVERS

OBSERVER	ANGLE READINGS IN DEGREES ( $\pm 0.5^\circ$ ) FOR CONCENTRATIONS OF						
	$1 \times 10^{-11}$	$1 \times 10^{-10}$	$1 \times 10^{-9}$	$1 \times 10^{-8}$	$1 \times 10^{-7}$	$1 \times 10^{-6}$	$1 \times 10^{-5}$
Junior author .....	6.0	13.0	17.5	19.5	22.0	24.0	25.5
Senior author .....	18.5	34.5	46.5	65.5*	.....	.....	.....

\* Readings above  $60^\circ$  are of very doubtful value.

The readings of the junior author are considerably lower and those of the senior author considerably higher than those obtained by most observers for similar concentrations. Readings for the same concentrations are usually quite constant for an individual over a considerable period of time but may vary over longer periods. Thus the readings of the junior author decreased gradually during a period of two years while those of the senior author increased greatly after eye strain from too constant observing. The data in table I were obtained at a time when a reading of  $39^\circ$  by the junior author corresponded to a concentration of about  $1 \times 10^{-4}$ .

<sup>3</sup> Readings for  $K_3PO_4$  were made because it has been impossible to find minima for

preparing the suspension and again after the algae had been in contact with the solution for an hour or more. A final reading was usually made a day or two later.

It may be seen in table I that part of the orthophosphate was converted into the meta form. The final readings for the metaphosphate were usually higher than those for the orthophosphate when the relative amounts of algae

TABLE I

TRANSFORMATION OF ORTHO- TO METAPHOSPHATE WITHIN THE ALGAL CELL  
(Angle readings vary directly with the concentration)

TEST NUMBER	ANGLE READINGS ( $\pm 0.5$ ) IN DEGREES*					
	INITIAL		AFTER 1 TO 3 HOURS		AFTER 1 TO 2 DAYS	
	$K_2PO_4$	$KPO_3$	$K_2PO_4$	$KPO_3$	$K_2PO_4$	$KPO_3$
1 .....	28.5	9.5	15.0	21.0	14.5	15.5
2 .....	37.0	9.5	28.5	14.5	16.0	18.5
3 .....	27.0	8.0	17.0	16.5	.....	.....
4 .....	22.0	8.0	7.5	14.5	6.0	8.0
5 .....	22.0	7.5	12.0	13.0	9.0	16.5
6 .....	20.0	9.0	12.5	18.0	5.5	16.5
7 .....	31.5	9.0	29.5	12.5	26.5	12.0
8 .....	36.5	12.5	33.0	13.5	26.0	9.5
9 .....	28.5	12.0	23.5	13.0	23.0	9.0

\* Observations by junior author. See footnote page 200.

and phosphate were such that the phosphate was rapidly disappearing. When, however, the amount of phosphate as compared with the algae was greater, the amount of metaphosphate appeared to hold a fairly constant level and the orthophosphate disappeared more slowly.

A second study was made to determine if pyrophosphate appeared as an intermediate form between the ortho- and metaphosphates. Algae used for this work had been growing for a long period without the addition of phosphate to the culture solutions. Suspensions were made in solutions of the composition previously stated. Examinations of the suspensions showed no ortho- or pyrophosphate. Metaphosphate was present in the suspensions made up on the first two days but it could not be detected in those made up later. Three aliquots of the suspension, two with algae and one from which the algae had been filtered were used each time. Orthophosphate was added to one of the unfiltered aliquots and to the filtered aliquot. Examinations were made before and just after the addition of the phosphate, and again after an hour or more. Final readings were usually made the following day.

salts of  $HPO_4^{--}$  or  $H_2PO_4^-$ . When such salts are dissolved, minima are obtained for the salt of  $PO_4^{---}$  (the amount of phosphate determined being equivalent to the amount of the particular cation present unless this cation had been added in excess of the phosphate), the hydroxide of the cation, and  $H_3PO_4$ . In this work an excess of the cation of the salt investigated was always present.

TABLE II

RELATIONSHIP OF ORTHO-, PYRO-, AND METAPHOSPHATES WITHIN THE ALGAL CELL  
(ANGLE READINGS VARY DIRECTLY WITH THE CONCENTRATION)

TEST NUMBER	TYPE OF SOLUTION	ANGLE ( $\pm 0.5$ ) READINGS IN DEGREES*										
		INITIAL			AFTER 1 HOUR			AFTER 2 OR MORE HOURS				
		PO <sub>4</sub> ----	P <sub>2</sub> O <sub>7</sub> -----	PO <sub>3</sub> -	PO <sub>4</sub> ----	P <sub>2</sub> O <sub>7</sub> -----	PO <sub>3</sub> -	PO <sub>4</sub> ----	P <sub>2</sub> O <sub>7</sub> ----	PO <sub>3</sub> -	PO <sub>4</sub> ----	P <sub>2</sub> O <sub>7</sub> ----
1	<div><div>Without PO<sub>4</sub>.....</div><div>With PO<sub>4</sub>.....</div><div>With PO<sub>4</sub>.....</div><div>(filtered).....</div></div>	0-† 44+ 44+	0- 0- 0-	43.5 43.5 0-	0- +† +†	0- 14.5 0-	43.5 46.0 0-	..... ..... .....	..... ..... .....	..... ..... .....	..... ..... .....	..... ..... .....
2	<div><div>Without PO<sub>4</sub>.....</div><div>With PO<sub>4</sub>.....</div><div>With PO<sub>4</sub>.....</div><div>(filtered).....</div></div>	0- 59.5 59.5	0.5‡ 0- 0-	19.5 19.5 0-	0- 57.5 59.5	0- 26.0 0-	19.5 24.0 0-	0- 56.5 59.5	0- 27.0 0-	19.5 29.5 0-	..... ..... .....	..... ..... .....
3	<div><div>Without PO<sub>4</sub>.....</div><div>With PO<sub>4</sub>.....</div><div>With PO<sub>4</sub>.....</div><div>(filtered).....</div></div>	0- 60.5 60.5	0- 0- 0-	0- 0- 0-	0- 59.5 60.5	0- 11.0 0-	0- 10.0 0-	0- 55.0 60.0	0- 25.5 0-	0- 44.5 0-	..... ..... .....	..... ..... .....
4	<div><div>Without PO<sub>4</sub>.....</div><div>With PO<sub>4</sub>.....</div><div>With PO<sub>4</sub>.....</div><div>(filtered).....</div></div>	0- 60- 60-	0- 0- 0-	0- 0- 0-	0- 60+ 60-	0- 20.0 0-	0- 20.0 0-	0- 60+ 60+	0- 46.0 0-	0- 50.0 0-	..... ..... .....	..... ..... .....

\* Observations by senior author. See footnote page 200.

† 0-, minima not seen at 0°.

‡ With PO<sub>4</sub> present, but angle reading not determined.

§ Probably contamination in tube.

Pyrophosphate soon appeared after the addition of orthophosphate to the suspensions and persisted over the period in which the determinations were made. The amount present shortly after the addition of the orthophosphate was sometimes greater than that of the metaphosphate, but later determinations always showed an amount of metaphosphate greater than that of the pyrophosphate. The results of these tests are shown in table II.

### Discussion

The transformation of orthophosphate to the meta form and the persistence of metaphosphate after the ortho and pyro forms could no longer be detected indicates that the meta form is important in the metabolic processes of algae. Some of the phosphate in biological material determined by certain investigators as pyrophosphate may have originated from the meta form since their methods did not distinguish between the two. According to WEISSFLOG and MENGDEHL (19), meta- and pyrophosphates are rapidly transformed into the ortho form after they are absorbed by corn plants. The fact that they found no meta- or pyrophosphate in the stems and leaves may have been because the amounts were too small to be determined by their method. MENGDEHL (13) was able to determine meta- and pyrophosphate which he added to plant material. It seems, therefore, unlikely that if considerable amounts were present in the material from corn plants used by WEISSFLOG and MENGDEHL (19) they would have been converted to the ortho form by the time the determinations were made. The results in table II show an amount of pyrophosphate greater than that of the meta form only for a time after the orthophosphate had been added to cultures deficient in phosphate. Algae grown for several months with adequate phosphate and then suspended in a solution of the composition given above also showed metaphosphate to be present in greater amounts than the pyrophosphate. The circle readings were as follows:  $\text{Mg}_3(\text{PO}_4)_2$ ,  $50^\circ$ \*;  $\text{Mg}_2\text{P}_2\text{O}_7$ ,  $26.5^\circ$ ; and  $\text{Mg}(\text{PO}_3)_2$ ,  $41.5^\circ$ . Examinations of the solution before the addition of the algae showed no meta-, ortho-, or pyrophosphate. These results indicate that pyrophosphate might be present only as an intermediate step between the ortho and meta forms.

### Summary

Living algal cells were examined by means of the ALLISON magneto-optic apparatus for the presence of meta- and pyrophosphates. These were found to be present except where the algae had been in phosphate-deficient media for a long time. Metaphosphate was present after the ortho and pyro forms could no longer be detected. The transformation of ortho- to metaphosphate

\* Readings not made above  $51^\circ$ .

and the persistence of the metaphosphate indicates that this form is important in the metabolic processes of algae.

ALABAMA POLYTECHNIC INSTITUTE,  
AUBURN, ALABAMA

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## BRIEF PAPERS

### PREPARATION OF INULIN FOR USE IN ADSORPTION COLUMNS

H. A. SPOEHR

Inulin has been found to be a very satisfactory material for the separation of the two chlorophyll components by means of the chromatographic adsorption method of TSWETT. Inulin has certain advantages over powdered sucrose for this purpose: it is less hygroscopic than sucrose; it apparently has a higher adsorptive capacity, so that smaller quantities and hence smaller columns are needed; the chlorophyll separates into two bands of the two components readily and these are easily eluted. Moreover, it is not necessary to remove the carotenoid pigments from the solution before adsorption, so that leaf extracts in petroleum ether solution can be used directly on the inulin adsorption columns.

The method which has been used for the preparation of inulin is in principle the same as that described by KILIANI (1) and which has recently been used by SPOEHR and MILNER (2, 3) for the isolation of leaf starch from plant materials. The source for the preparation of inulin has been dahlia tubers which were obtained as culls from nurseries.

The dahlia tubers were scrubbed to remove all soil, and decayed and woody portions were cut out. The tubers were then cut into slices of a convenient size to feed into a mincer through which they were passed three or four times, using a medium-sized cutter. This was found to be better than to mince the material very fine, because of the loss of juice with the fine mincer. Batches of five kilos of the ground tubers, mixed with three liters of water and 25 gm. of powdered calcium carbonate were boiled vigorously for one-half hour. The hot mixture was filtered with suction through muslin on a large Buchner funnel and the pulp thoroughly pressed out on the funnel. The pulp in the funnel was then washed with 500 cc. of hot water and thoroughly drained by suction and pressure. A second extraction of the pulp hardly repays the time and effort required. The hot filtrates were immediately filtered again through large folded filters to produce a perfectly clear, dark brown solution.

The solutions obtained in the manner just described were then subjected to freezing. This was done in five-gallon ice cream cans, which were stored at  $-20^{\circ}$  C. for 5 days. The solutions were then allowed to thaw at room temperature. The inulin remained insoluble and was removed by filtration through filter paper on a Buchner funnel under reduced pressure, or preferably on large sintered Jena glass filters. The phenomenon is very similar to that of retrogradation of starch (2, 3). Incidentally it may be mentioned that it has been found impossible to separate glycogen from aqueous solution

by this method. The filtration of the inulin may become a rather tedious process and it was found advantageous to filter the main portion with only slightly reduced pressure in order to avoid clogging the pores of the filter. But if the inulin solution has been kept frozen for some days and the filtration is done cautiously, it proceeds fairly rapidly. The moist inulin is very susceptible to infection by molds, so that it should be worked up immediately.

The inulin was then dissolved again in hot water, filtered, and the solution frozen at  $-8^{\circ}\text{C}$ . The volume of solution in this step was very materially less, about one-tenth of that used in the first extraction, and the freezing process was carried out in ice trays of a household electric refrigerator. After several days, the solutions were allowed to thaw and the inulin was filtered on sintered glass filters with suction, with the precautions already mentioned. The inulin was dried in an oven at  $60^{\circ}$ , coarsely ground, and dried in vacuum over calcium chloride for several days. It was then powdered in a pebble mill, passed through a 200-mesh sieve, and dried in vacuum over phosphorus pentoxide.

The inulin thus prepared was pure white; different preparations had an ash content of 0.16 to 0.20 per cent. and a specific rotation of  $[\alpha]_{\text{D}}^{20} = -35.3^{\circ}$  to  $-38.1^{\circ}$ . From one lot of 27.8 kilos of dahlia tubers, which were extracted in six batches, there were obtained first crops totaling 1640 gm. of inulin, *i.e.*, a yield of 5.8 per cent. The filtrates from the second freezing were combined, concentrated, decolorized with charcoal and frozen again, and yielded an additional 85 gm. of inulin, making a total yield of 6 per cent.

One further advantage of the use of inulin is that it can be readily recovered after use as an adsorbent. It is washed with ethanol, dissolved in hot water, filtered, decolorized with charcoal, if necessary, and by freezing the solution it can be recovered in pure form as described.

CARNEGIE INSTITUTION OF WASHINGTON  
DIVISION OF PLANT BIOLOGY  
STANFORD UNIVERSITY, CALIFORNIA

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## EFFECTS OF X-RAYS ON SEEDS

A. A. BLESS

In a recent paper (1) the author has shown that seeds subjected to various doses of x-rays show peculiarities at certain stages of the growth which are not necessarily maintained throughout the period of growth, and cannot be used as a criterion for the determination of the best dosage for yield at maturity.

In order to obtain some information concerning the effects of x-rays on seeds, by examination of mature plants from x-ray treated seeds, experiments were made on seeds of radishes, lettuce, and beans. The x-ray tube was operated at about 100,000 volts and 5 milliamperes. The rays were filtered through 1 mm. of aluminum, and the seeds were placed at a distance of 30 cm. from the target. They were subjected to the radiation for various lengths of time. The growth of the seeds was recorded every week, and showed phenomena similar to those exhibited by corn in the paper mentioned above and in agreement with the work of JOHNSON (2) and will not be discussed here.

According to SHULL and MITCHELL (3) the effects due to the x-rays are more pronounced when the seeds are subjected to the radiation in sprouted condition. In order to test this hypothesis two sets of experiments were conducted; one set of seeds was exposed to x-rays when dry, while the other set was exposed when the seeds were sprouted. For the latter set the seeds were allowed to remain in trays on sterilized cotton padding saturated with distilled water until definite sprouts appeared and then subjected to x-ray treatment. The results of the treatment are shown in table I.

The results show that the x-ray treatment did not produce significant changes in the case of radishes. The improvements in weight of the plants from treated seeds as compared with those from untreated is not very large, and may therefore be attributed to accident. Moreover there is no significant difference between the weight per pod of the seeds exposed when sprouted and those exposed when dry. The increase on the average is only about 10 per cent. which cannot be considered to be very important.

In the case of beans, plants from seeds treated dry have shown no significant changes over the untreated. However, plants from the irradiated sprouted seeds have shown increases of some 25 per cent. over the controls, which is probably too large to be considered accidental.

The results were more interesting in the case of lettuce. While the increase in weight was not significant in the case of most of the plants produced from seeds treated in the dry state, the five-minute dose produced an increase in growth of about 60 per cent. over the controls. Because of this large increase, the experiments were repeated a year later. The results indicated

TABLE I

PLANT	TIME OF EXPOSURE	EXPOSED DRY		EXPOSED SPROUTED		REMARKS
		WEIGHT PER PLANT		WEIGHT PER PLANT		
	<i>min.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	
Radishes, (Cincinnati Market)	0	3.3		4.0		In all 250 plants; about 20 plants in each set
	$\frac{1}{4}$	4.3		5.3		
	$\frac{1}{2}$	2.0		2.4		
	$\frac{3}{4}$	5.3		4.8		
	1	3.5		4.5		
	2	4.8		5.7		
	3	2.7				
	4	4.5		4.4		
	7	4.5				
Beans (Bountiful)	0	6.2		5.3		300 pods; about 25 in each set
	$\frac{1}{4}$	4.8		3.8		
	$\frac{1}{2}$	5.1		8.2		
	1	6.4		6.9		
	2	6.2		5.3*		
	4			7.3		
	6			5.5*		
		1936	1937	1936	1937	
Lettuce (White Boston)	0	4.3	4.0	4.3	5.0	300 plants; about 10 in each set
	$\frac{1}{4}$	4.7		6.0		
	$\frac{1}{2}$	3.7		3.8*		
	$\frac{3}{4}$	3.9		2.0*		
	1	4.7	4.0	5.1	6.9	
	2	4.0	6.65	5.8	5.7	
	3	3.6	4.5	4.7		
	4		6.0		8.6	
	5	7.0	6.1		7.7	
	6		4.9	5.0	6.1	
	7	3.4	4.7		8.1	

\* Poorly sprouted.

that for the 4- and 5-minute exposures the increase is well over 25 per cent. In the case of the seeds irradiated when in sprouted condition, the increase in weight of plants grown from the treated seed over the controls was on the average about 50 per cent. for the larger doses used.

In addition to the differences in weight the x-rays seem to have also caused a noticeable difference in taste. About half of the people who have tasted the lettuce in 1936 have been able to detect this difference. The lettuce seemed to have a stronger, "tangier" taste, the "tanginess" increasing with the dose. In 1937 eight people out of twelve who tasted the lettuce have observed this difference. Considering the fact that the sense of taste varies a great deal with different individuals, the difference introduced by x-rays is probably real.

In addition to these greenhouse experiments, x-rayed seeds of corn were planted in the field. For these experiments, an inbred variety developed

in the Florida Agricultural Experiment Station was used. The seeds were planted in the field and so staggered as to minimize the effects of possible soil variation. The inbred variety, while possessing the advantage of small individual variation, possessed the great disadvantage of lower resistance to diseases and to the ravages of the corn borer. In place of the two hundred ears of corn expected for each batch, only about 40 per cent. of the normal crop was obtained. The results are presented in tabulated form.

TIME OF EXPOSURE IN MINUTES (EXPOSED DRY)	WEIGHT IN GRAMS PER EAR
0	204
1	213
2	198
4	213
8	212

It appears that for two or three doses, there is a small increase in weight of the ears grown from the x-rayed seeds over the controls. The result cannot be considered very significant.

The results of these experiments may be summarized briefly as follows: within the limits of the x-ray doses used in these experiments, significant effects of x-ray on the yield at maturity have been found only for lettuce and for beans. The response to the x-ray treatment was greater when the seeds were in sprouted condition. No significant changes have been observed in the case of radishes, exposed sprouted as well as dry, and of corn exposed dry.

It gives me great pleasure to acknowledge the advice and the generous cooperation which has been given to me by Dr. F. S. JAMIESON and Dr. F. H. HULL, of the Florida Experiment Station.

UNIVERSITY OF FLORIDA,  
GAINESVILLE, FLORIDA

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## NOTES

**Indianapolis Meeting.**—In many ways the fourteenth annual meeting of the American Society of Plant Physiologists was the most satisfactory meeting in its history. The arrangements left little to be desired, and more was accomplished for the future welfare of plant physiology than at any previous meeting. Space does not permit publication of details of the actions taken, but they will vitally affect the financial background of the Society's program. The program committee, through the efforts of its chairman, Mr. WITHROW, and with the able assistance of all members of the committee, developed a series of meetings that set a high standard of excellence. The annual dinner was attended by about 175 individuals. As usual, the important features of this gathering were the STEPHEN HALES address, *Hormones and the Analysis of Growth*, by Dr. K. V. THIMANN, and the announcements of awards and honors. The officers of the Society, and its committees responsible for the success of the meeting, deserve great credit for the high standards set in the performance of their duties. We are indeed fortunate in having so many capable and efficient servants.

**Annual Meeting, 1938.**—The next annual meeting of the Society will be held at Richmond, Virginia, in connection with the A.A.A.S. meetings in December, 1938. The personnel of the program committee, as well as that of the program committee for the summer meeting at Ottawa, Canada, late in June, will be announced in April. Early consideration of symposia subjects and joint meetings is advisable so that more careful preparation can be made by those participating in the discussions. In addition, careful consideration should be given in advance to such problems as hotel and meeting room requirements in order that the most favorable arrangements may be made when the assignments are in order.

**Life Membership Awards.**—The BARNES Life Membership committee had been authorized to make two awards at Indianapolis, if it was found feasible to do so. In accordance with this authorization, which followed a precedent set at the Des Moines meeting in 1929, the committee, with Dr. H. R. KRAYBILL as chairman, presented the names of two candidates for the awards. The two men chosen for this high honor were Dr. HOMER LEROY SHANTZ, of the U. S. Department of Agriculture, and Dr. ALEXANDER P. ANDERSON, Tower View Laboratory, Red Wing, Minnesota. Brief biographies of the two candidates are given.

Dr. A. P. ANDERSON was born in Red Wing, Minnesota, on November 22, 1862. His collegiate training was obtained at Minnesota, where he received the B.S. degree in 1894, and the M.S. degree in 1895. As was customary at

the time, he went to Germany for his Ph.D., which was granted at Munich in 1897. He was botanist and bacteriologist at Clemson College just after his return from Europe, assistant professor of botany at Minnesota for two years, once more biologist and entomologist at Clemson during 1900-1901, and curator of the herbarium at Columbia University for a short period. After this he went into private research, which took an industrial turn, and to this work he has given a lifetime of service. He was included among the first group of 100 botanists to be starred in the first edition of *American Men of Science*.

His father, JOHN ANDERSON, and his mother BRITTA (GUSTAFSDOTTER) ANDERSON, were both natives of Sweden. He married LYDIA JOHNSON, of Highlands, North Carolina, shortly after returning from Munich. Five children have graced their home.

Dr. ANDERSON is best known for his invention of puffed cereals, which came out of his early work on processing of starch and cereal products. Puffed rice, puffed wheat, Quaker crackels, and other puffed products we owe to his inventive genius. But before he went into these industrial problems, he was interested in the formation of abnormal resin canals in the Coniferae, in the growth of fruits as determined by weighing, and in plant diseases. He has on various occasions shown his interest in the work of the American Society of Plant Physiologists, as a contributor to the STEPHEN HALES fund in 1927, and as a patron of the Society. He passed his 75th birthday anniversary in November, 1937. The action of the committee in making the award is very appropriate.

Dr. H. L. SHANTZ was born in Kent Co., Michigan, on January 24, 1876. He received the B.S. degree from Colorado College in 1901, and the Ph.D. degree from the University of Nebraska, in 1905. He had had experience in the teaching of botany at Colorado College, and at Nebraska, during his graduate training. After obtaining his doctorate, he served for short periods at Missouri, and at Louisiana. In 1908 he joined the staff of the U. S. Department of Agriculture, and was especially interested in the problems of alkali and drought resistance, and the breeding of resistant races. He was plant physiologist in the Bureau of Plant Industry from 1910 to 1926, and in charge of plant physiology and plant geography during the last six years of this period. During 1926 to 1928 he was Professor and head of the Department of Botany at Illinois, from which institution he went to the University of Arizona as president in 1928. Recently he has returned to the Department of Agriculture.

In 1901 Dr. SHANTZ was married to LUCIA MOORE SOPER. They have two sons. In connection with his varied services, Dr. SHANTZ was a traveler and explorer. He went with the Smithsonian Expedition to Africa in 1919-1920, and was a member of the Educational Commission to East Africa under the Phelps Stokes Fund and the International Educational Board in 1924.

His name is best known among plant physiologists and ecologists for his work on the water relations of plants and soils, the wilting coefficient, the moisture equivalent, the water requirement of plants, etc., and for his studies of the indicator value of native vegetation in the Great Plains area. These investigations, however, represent but a small part of his scientific contributions which have been many and varied.

The American Society of Plant Physiologists is greatly honored by the addition of the names of these two eminent men to its roll of CHARLES REID BARNES life memberships.

**Corresponding Members.**—During the year 1937 two corresponding members of the American Society of Plant Physiologists were lost by death. At the Indianapolis meeting, therefore, two new members were elected. The two plant physiologists chosen for these honors were Dr. HENRY H. DIXON, of Trinity College, Dublin, Ireland, known throughout the world for his contributions to the problems of ascent of sap, particularly for the cohesion theory of sap rise, and Dr. ALFRED URSPRUNG, of Fribourg, Switzerland, known best for his accurate measurements of osmotic pressure. To these men American plant physiologists extend cordial greetings and best wishes. The Society is happy to honor these distinguished men for their great contributions to plant physiology.

**New England Section.**—The New England Section is planning its annual spring meeting for May 3 and 4, 1938. The meeting is to be held at Orono, Maine, under the auspices of the University of Maine. In addition to a session for short papers for Friday afternoon, May 3, there will be a symposium on the physiology of aquatic plants. Participants in this symposium will be Dr. F. H. STEINMETZ and Dr. G. P. STEINBAUER, of the University of Maine, Dr. W. C. MUENSCHER, Cornell University, and Mr. CLARENCE COTTAM, of the U. S. Biological Survey. The annual dinner will be held as usual on Friday evening, with after dinner discussions. These New England Section meetings have been very well attended in the past, and it is hoped that all members of the section will plan to attend this meeting at Orono.

**Endowments.**—The plans which have been developing over a number of years for the gradual increase of the general endowment of the American Society of Plant Physiologists have now been completed and put into operation. The final steps were taken at Indianapolis. In a few years it will be possible to estimate the average productivity of the plan as a whole. During 1937 the general endowment was enlarged from somewhat over \$700 to about \$1200. Much of this growth was obtained through gifts of patrons, but there will be steady growth in the future due to other sources of endowment.

income. It is hoped that the steady increase in the number of patrons may continue. Is there any more enduring benefit to mankind which one can leave behind him than the possibility of continuously increasing knowledge? Those who become patrons are helping to make more certain the perpetual enrichment of human life through scientific investigation. Gifts are always carefully safeguarded, and the need challenges conscientious thought on the part of all scientists as well as laymen.

**Patrons.**—At the Atlantic City meeting in 1936 the first patrons of the Society were elected. Two more were added at the Denver meeting in June, 1937, and another was added at Indianapolis. The latest addition to the ranks is Professor FRANK M. ANDREWS, for many years plant physiologist at Indiana University. It is gratifying to know that the aims and purposes of our organization are appealing to so many people in terms of permanent support. Professor ANDREWS has conferred a great honor upon the Society by this additional evidence of his loyalty, devotion, and generosity toward it, a loyalty and devotion which has been expressed in many other ways during the last 15 years. It is a pleasure to record, and to express to him our appreciation of his generous aid.

**Bohumil Němec.**—During the last year we have attempted to bring closer to American workers the great personalities of our corresponding members. In this number we pay our tribute of honor and respect to Dr. BOHUMIL NĚMEC, who reaches his 65th birthday anniversary on March 12, 1938. On behalf of all American plant physiologists, we extend to him our cordial greetings and good wishes for that happy occasion, and wish him long years of usefulness in the field to which he has contributed so ably. Inasmuch as the story of the progress of plant physiology in Czechoslovakia is so closely identified with the life of Professor NĚMEC, we are not attempting any additional biographical sketch at this time. We are greatly indebted to Dr. PRÁT for his assistance with the photograph which is reproduced in this issue of PLANT PHYSIOLOGY, and to Professor FRANK M. ANDREWS through whose efforts the original was rephotographed to improve the reproduction.

**Hans Molisch.**—Dr. HANS MOLISCH was born July 12, 1856, and was therefore 81 years old at the time of his death on December 8, 1937. For a good many years he had been professor of botany at the University of Vienna. He had also been professor of botany at Praha and at Sendai, Japan. A good part of his research has been done in the United States, in Java, and also in India. At the University of Praha he was director of the Plant Physiological

Institute and later also held the same position in the University of Vienna. He was the author of about 200 scientific contributions many of which were in the form of books. It would take us too far to enumerate the volumes he has written. He always wrote in a very interesting way and with remarkable clearness. He succeeded WIESNER and during the last 50 years he has been busily engaged in his scientific investigations. His plant physiological investigations have influenced almost every phase of this science. Many of his books are absolutely indispensable to the plant physiologist. Among these may be mentioned his *Mikrochemie der Pflanzen* which covers a field in which he made many valuable contributions. His correspondence was very large but nevertheless he, as was true of many other great men, always found time to write his letters in long-hand. Professor MOLISCH travelled extensively and wrote several volumes concerning these travels. He was a keen observer and always wrote just as interestingly of his travels as of his direct investigations. On one of these trips he visited the United States. Professor MOLISCH has been the recipient of many honors; during his 80th year he was vice-president of the Academy of Science of Vienna, and at the time of his birthday was highly honored and received distinction from many countries. In 1935 Professor MOLISCH was elected a corresponding member of the American Society of Plant Physiologists. Those who knew and read after Professor MOLISCH will miss him greatly. He was one of the most famous plant physiologists and in his death science has suffered an irreparable loss.—Memorial Committee.

**Upendra Kumar Das.**—Plant physiology has suffered another serious loss in the death of Dr. U. K. DAS. He was born in Habiganj (Assam), India, July 22, 1902. He attended the University of Hawaii where he received the B.S. degree with honors in 1927 and the M.S. in 1930. From 1927 to 1934 he was Research Agriculturist in the Hawaiian Agricultural Experiment Station at Honolulu. He received the Ph.D. degree from the University of Minnesota in December, 1935. Although just beginning his research work his publications number 15 in the Hawaiian Sugar Planters' Record. His thesis on *Nitrogen Nutrition of Sugar Cane* was published in PLANT PHYSIOLOGY 11: 251-317. 1936. Due to an explosion of apparatus in his laboratory he was instantly killed on October 22, 1937. He possessed a thoroughly scientific attitude of mind and was eminently practiced in all his work. He will be greatly missed by all of his associates and others as well. His death is a great loss to plant physiology and to replace him will be difficult.—Memorial Committee.

**Chronica Botanica.**—This chronicle of botanical progress has been re-organized, and becomes a bi-monthly journal instead of a year-book with the

issues of 1938. The subscription price has been reduced from 15 to 7 guilders. The aims of the new journal continue those of the former yearbook, with the addition of publication of brief papers and discussions. Its purposes are to promote documentation, good will, and international cooperation among plant scientists. There are eight other sections provided in the new journal as follows: scientific communications, forum botanicorum, international congresses, quotations, miscellaneous news, personalia, queries, and new periodicals. Those interested in possession of the new journal, individuals or librarians, or those who wish to use its facilities in the publication of scientific notes, discussion, or news are invited to correspond with FR. VERDOORN, P.O. Box 8, Leiden, Holland.

**Agrobiology.**—*The ABC of Agrobiology* is the title of a recent book by O. W. WILLCOX, who has written several works in which he intimates that agriculture, pursued in the fulfillment of certain mathematical equations, would be enormously more productive than it is now. There are 18 chapters in this new venture, enough, almost, for an A to Z presentation.

If all limiting factors could be lifted from plant growth, and if the growth capacity of all agrotypes could be built up to theoretical maxima by genetic improvement, of course we would have larger yields. But this would require perfect climatic conditions, as well as perfect soil conditions, both of which are unattainable in any very considerable area under cultivation. Mathematical equations look like accuracy and precision, but they do not help one if he lives in a dust bowl! Furthermore, WILLCOX assumes that the percentage of nitrogen which plants will build into their bodies is genetically controlled and constant for any given agrotype. He figures out that no plant type can take over 318 pounds of nitrogen from an acre of soil. By dividing percentage of nitrogen content into this value, he computes theoretically possible yields.

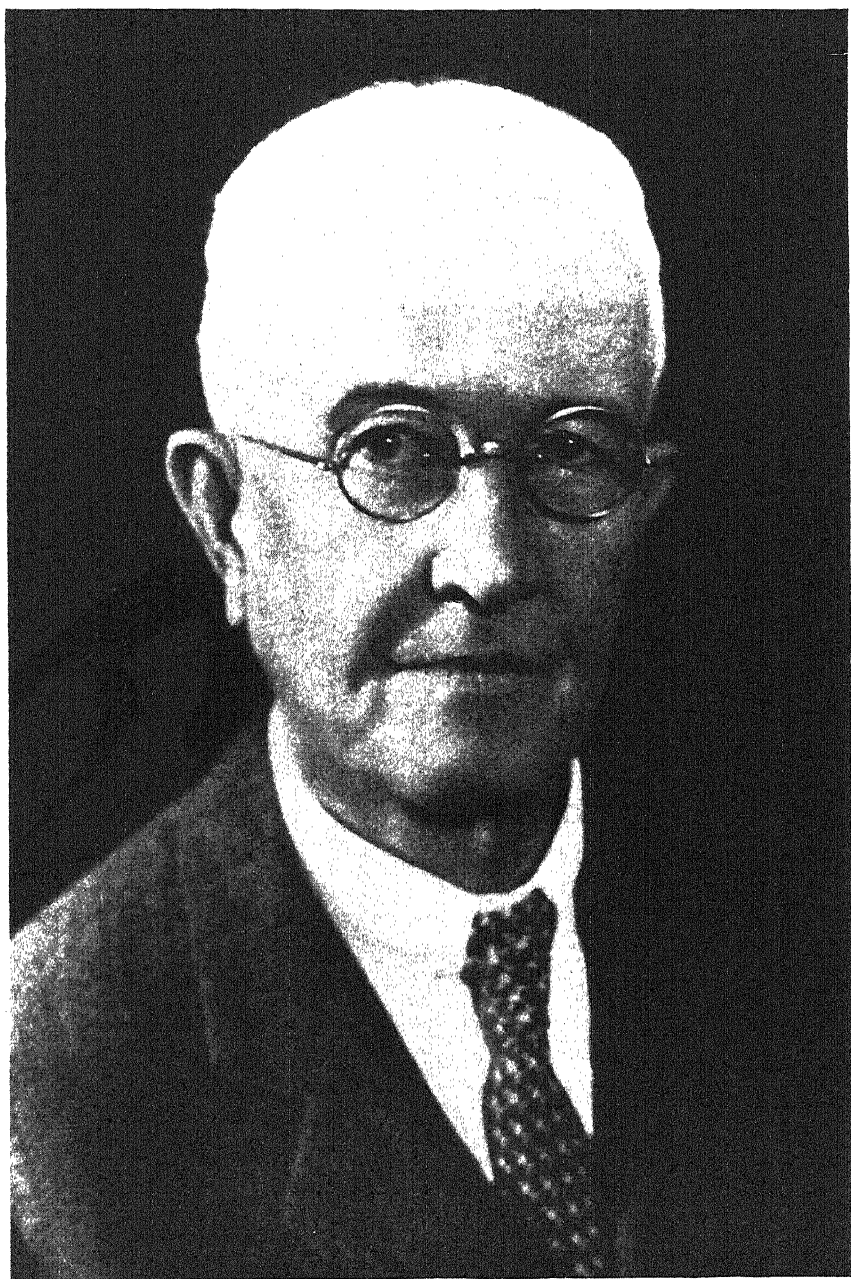
Against these assumptions, one must place the known facts that one can vary the amount and kind of nitrogen present in plants almost at will; that there is no constancy in the nitrogen relations; that maximum nitrogen intake often means decidedly low yielding capacity; that the quality of fruits and vegetables may be ruined by too much nitrogen, etc. The experienced grower will have no difficulty in detecting many of the errors involved in WILLCOX's reasoning.

The author evidently intended this book to be elementary enough to assist the lay-gardener, orchardist, and general farmer. Unfortunately there does not seem to be much chance that it will help any of them, because it does not solve any of their real problems. The book contains 323 pages, is offered at \$2.75 per copy, and is published by W. W. Norton & Co., New York.



THIS NUMBER OF PLANT PHYSIOLOGY  
IS DEDICATED TO  
GEORGE JAMES PEIRCE  
IN CELEBRATION OF  
THE SEVENTIETH ANNIVERSARY OF HIS BIRTH  
MARCH 13, 1868





GEORGE JAMES PEIRCE  
MARCH 13, 1868



# PLANT PHYSIOLOGY

APRIL, 1938

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## PRELIMINARY EXPERIMENTS ON THE RELATION OF GROWTH-PROMOTING SUBSTANCES TO THE REST PERIOD IN FRUIT TREES

J. P. BENNETT AND FOLKE SKOOG

(WITH ONE FIGURE)

### Introduction

Prolonged rest of leaf and flower buds of fruit trees occurs commonly in mild climates as in the western and southern states. It is especially evident in springs following mild winters, but may be nearly absent in springs following more severe winters.

Similarly it has been found that the rest period can be artificially prolonged by keeping plants under warm conditions in a greenhouse or can be artificially broken by placing the trees in cold storage (2). Many other treatments have been used for breaking the rest, but the action of these diverse agents has given no clue to the nature of the physiological factors responsible for the rest condition. Work on inhibition of lateral buds on plants has shown that the growth-promoting hormone, auxin, is a controlling factor. There are several reasons for believing that also in the resumption of growth by buds of trees, growth-promoting substances are involved. In this article results are presented of preliminary experiments undertaken from this viewpoint.

### Experimentation

#### DETERMINATION OF AUXIN IN LATERAL BUDS OF PEAR AND CHERRY

The presence and distribution of auxin in lateral buds of trees has been studied by ZIMMERMANN (5). The change in auxin concentration in terminal buds of apple during the growing season has been followed by AVERY, BURKHOLDER, AND CREIGHTON (1). In the present work, resting lateral buds in which no hormone could be obtained by the *standard Avena* technique, were studied by means of the *deseeded Avena* test method (4). In November, buds from pear and cherry trees growing in the orchard were tested for auxin. The buds were cut at the base of the scales, placed on wet

filter paper for 10 to 20 minutes and then individually on standard 2 per cent. agar blocks for two hours. The blocks were then tested by the standard and also by the *deseeded Avena* method. No trace of *active auxin* was found. At this time, two-year-old cherry and pear trees were placed in cold storage at a constant temperature of 2° C. and comparable trees were moved into a greenhouse at a minimum temperature of approximately 15° C. At intervals buds from each of a number of previously selected comparable branches on the different trees were tested for the presence of auxin.

The results are summarized in table I. For small concentrations of auxin, curvatures reach a maximum not later than 4 to 5 hours after the blocks are applied. After longer times of application, traces of activity comparable to those produced by substances which can be converted into auxin were obtained. It can be seen that in the first four tests no curvatures characteristic of auxin were obtained. The next three experiments indicate that such a substance accumulates in the buds in cold storage but not in those in the greenhouse. The later experiments show that after an extended exposure to low temperature either in cold storage or in the field the buds are able to cause relatively large characteristic auxin curvatures in deseeded plants and also give significant curvatures in standard tests. From buds in the greenhouse, however, no response could be obtained. Soon after the appearance of auxin an increase in size of the buds was observed. In the tests made March 4 and 5, cherry buds from cold storage had leaves protruding about 1 mm. above the scales. The buds of cherry and pear from the field, tested March 4, were slightly more developed, as were the cherry buds from cold storage in the last test, May 3. It is realized that many metabolic changes occur during breaking of the rest period. The liberation of auxin in the buds may be one result of these changes essential for resumption of growth.

#### BREAKING OF THE REST PERIOD BY ADDED GROWTH-PROMOTING SUBSTANCES

The results presented above indicate a possibility of breaking prolonged rest periods of buds by the administration of growth-promoting substances. Resting one-year-old pear, apple, and peach trees grown continuously in a warm greenhouse were used. Tips were removed from selected branches and dilute aqueous solutions of the substances listed in table II were passed into the trees through the cut ends. The effect of the treatments on the development of buds was noticeable two to four weeks after application. The final observations, April 1, are summarized in table II. It can be seen that on trees receiving no treatment a few buds grew on two pear trees, one on two apple trees, and none on peach trees. In trees injected with water, usually one to three buds next to the cut end on the injected branch grew, but not vigorously. In trees injected with DN, tryptamine, or vitamin B<sub>1</sub> the number of developing buds was no greater than in those injected only with water. But the application of vitamin B<sub>1</sub> possibly caused more vigorous development

TABLE I

AMOUNTS OF AUXIN, IN DEGREES, OBTAINED FROM LATERAL BUDS PLACED ON AGAR BLOCKS FOR 2 HOURS, AS DETERMINED BY DESIGNED *Avena* METHOD. EACH FIGURE REPRESENTS THE MEAN OF TEN TO TWELVE BUDS. THE NEGATIVE VALUES REPRESENT AUXIN ACTIVITY

DATE	TIME OF TEST IN HOURS	CHERRY			PEAR			AGAR BLOCKS
		BUDS FROM TREES IN			BUDS FROM TREES IN			
		GREENHOUSE	COLD STORAGE	FIELD	GREENHOUSE	COLD STORAGE	FIELD	
11/23/36	2	+ 1.2	+ 1.6		-0.3	+ 1.8		
11/29/36	14	-2.0	- 3.0		-2.2	- 1.2		
	S.T.*	+	+	+	+	+	+	
	5			+ 1.2			+ 0.1	
12/1/36	5			+ 1.1			.....	
	18			- 3.0			- 2.2	
	18			- 3.2			.....	
12/9/36	15	-0.2	- 0.4		-0.1	+ 0.6		+ 0.1
12/23/36	18	-1.6	- 6.2		-3.8	- 4.5		+ 1.5
1/18/37	17	-0.5	- 5.5		-2.3	- 7.1		+ 2.0
2/12/37	18	.....	- 4.0		.....	- 4.0		
3/4/37†	5			-17.7(1)†			-18.8(2)†	
	5			-23.9(2)			-10.7(3)	
	5			-13.6(3)				
3/5/37	18	-0.2	- 2.6	- 3.0	-2.2	- 3.2	- 7.3	
	18	+0.2	- 3.3	- 2.0	-2.5	- 5.4	- 7.5	+ 0.9
	S.T.	+0.2	- 4.5	- 3.2	.....	- 5.6	- 6.1	
3/11/37	S.T.	+1.5	- 3.0	- 4.3	-2.0	- 9.2	.....	
	18	+2.8	- 2.9	- 6.3	-2.3	- 4.5	- 3.9	
	S.T.	+1.5	- 2.3	- 1.3	+0.8	+ 0.8	+ 2.5	
5/3/37	3.5	+1.4	-24.2		-0.2	- 4.8		
	24	0.0	.....	.....		-17.3		
	S.T.	+3.5	-12.4	.....		- 9.0		

\* In standard test, (S.T.) blocks are applied 110 min.

† Buds had grown to lengths of 0.5 to 0.8 cm.

‡ (1), (2), (3) refer to time in hours buds were left on agar blocks.

TABLE II  
EFFECT OF VARIOUS SUBSTANCES ON GROWTH OF DORMANT BUDS

TREES OBSERVED	SUBSTANCE		No. BUDS	No. BUDS GROWING APRIL 1	REMARKS
	APPLIED	AMOUNT			
Pear 12/1	Branch 1	Yeast ext. 1	31	2	Only apical buds grew
" "	" "	" "	42	4	
" "	" "	" "	36	21	4 buds near top developed shoots
" "	" "	" "	15	10	Branch killed but many buds on trunk above and below grew
" "	" "	" "	10.0 "		
Pear 2/22	Tree 1	Yeast ext. 2	175	49	Growing buds distributed over whole tree—12 on trunk
" "	" "	" "	149	37	Growing buds distributed over whole tree—13 on trunk
" "	" "	Vitamin B <sub>1</sub>	191	2	Only apical buds on injected branch grew
" "	" "	" "	137	4	" " " " " "
" "	" "	" "	191	6	" " " " " "
" "	" "	*D N	182	3	Growing buds on upper branches, 1 on trunk
" "	" "	Water	128	1	Apical buds on injected branches grew
" "	" "	0	174	6	
" "	" "	0	124	8	3 buds grew on trunk
" "	" "	Heteroauxin			Growing buds on injected or adjacent branches
Apple 2/19	Tree 1	Yeast ext. 2	51	30	Best growth on lower part. Upper part of tree injured
" "	" "	" "	50	6	Only lower buds grew. Upper part of tree killed
" "	" "	Vitamin B <sub>1</sub>	50	1	Growing bud near top
" "	" "	" "	50	3	" " buds " "
" "	" "	" "	48	2	" " " "
" "	" "	Water	50	0	
" "	" "	0	49	1	Growing bud near middle of trunk
" "	" "	0			

\* Dinitro-ortho-cyclo-hexyl-phenol.

TABLE II—(Continued)

TREES OBSERVED	SUBSTANCE		No. BUDS	No. BUDS GROWING APRIL 1	REMARKS
	APPLIED	AMOUNT			
Peach 1/5	Tree 1	Yeast ext. 2	54	34	4 branches injected 2 cc. each; lower buds grew best
"	" 2	" "	29	19	5 branches injected 2 cc. each; lower buds grew best
"	" 3	" "	32	14	Injected through top of trunk. Upper branches and trunk killed
"	" 4	" "	95	40	4 branches injected, 2 cc. each
1/20	" 5	Water	30	2	3 branches injected, about 10 cc. each
"	" 6	Tryptophane	45	8	Growth of buds weak
"	" 7	Tryptamine	74	0	Apical buds grew weakly
2/19	" 8	Vitamin B <sub>1</sub>	28	2	" "
"	" 9	" "	39	2	" "
2/22	" 10	Heteroauxin	54	21	Buds grew from tip to base of injected branch, moderately strong growth
"	" 11	0	420	0	

of the buds that grew. Tryptophane was applied to peach trees only. It caused weak growth of a number of buds along the injected branch.  $\beta$ -indoleacetic acid (heteroauxin) produced moderate growth of buds from tip to base of injected branches and some development near the bases of lower adjacent branches in peach trees, while in pear trees it caused moderate development of a few scattered buds on injected and adjacent branches.

The best development of buds was obtained by the injection of a preparation obtained by a series of extractions of autolyzed brewers' and bakers' yeasts. On treated peach trees the buds grew vigorously along the injected branches and on the lower parts of adjacent branches. In the treated apple trees which were relatively small and had only a few branches, the dosage was too large, so that the upper portions of the stems were damaged. Below the damaged regions, however, buds developed along the entire stems. On pear trees (fig. 1) the development of buds extended from the point of injection throughout the entire tree.

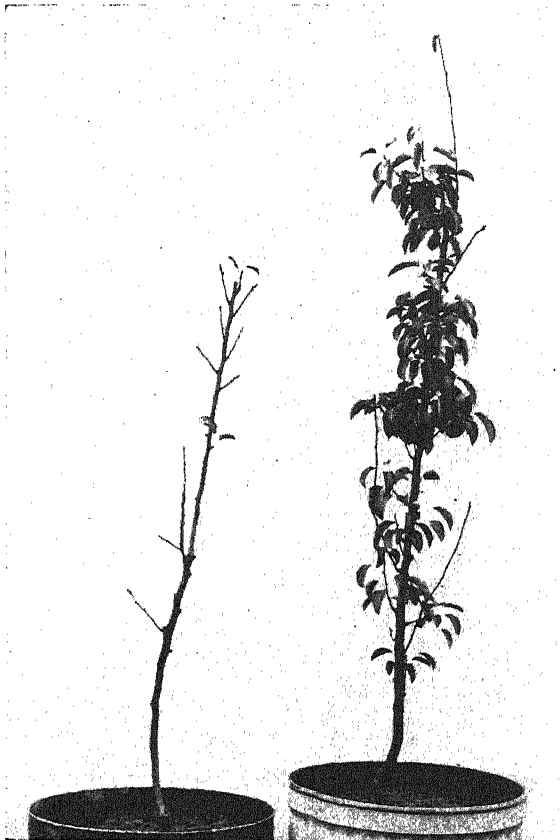


FIG. 1. The effect of injected yeast extract on Bartlett pear trees. Left, untreated; right, treated.



On the peach trees the developing shoots grew to lengths of 2 to 12 cm., on the pear trees, to lengths of 4 to 12 cm. and then ceased growing. On the apple trees, however, elongation continued until the shoots were 25 to 50 cm. long.

### Discussion

The results of these experiments demonstrate again that low temperature, either in the field or in cold storage, is essential for normal breaking of the rest period. As a result of exposure to low temperature a precursor of auxin accumulates in the buds, followed by the gradual appearance of auxin. Its appearance in the buds is correlated with ending of the rest. Our results are in striking agreement with the work of MIROV (3) who demonstrated that in resting seeds of sugar pine no active auxin is present, but that upon stratification in cold storage, auxin gradually appears in considerable concentrations, and that parallel with its appearance the seeds are able to germinate. The results presented here also show that rest can be broken by the administration of certain growth-promoting substances. Further experiments on a larger scale than as yet has been possible are necessary to determine the active component from yeast, and to determine the relative effectiveness and relationship of the different active substances. Merely arbitrary dosages were chosen above. The fact that heteroauxin in these experiments was found to stimulate the growth of resting buds is not to be considered contradictory to its known inhibiting action on bud development under different physiological and experimental conditions.

UNIVERSITY OF CALIFORNIA  
BERKELEY, CALIFORNIA

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# PLANT DIASTASE IN EVIDENCE AS TO THE FORMATION AND STRUCTURE OF STARCH GRANULES<sup>1</sup>

GEORGE L. TELLER

(WITH SEVEN FIGURES)

## Introduction

In a recent paper (3) evidence was presented as to the existence of two distinctive sugar-forming plant diastases. These diastases occur together in certain cereals and in various other plants; but in many plants only one of them is apparent. No reference was made in that paper to a third type predominately liquefying, which accompanies these two and which is essential to the purpose which they serve. This latter diastase can best be understood by stating the characteristics of the other two.

## Investigation

In a study of different parts of the wheat grain, weighed quantities of finely powdered material of each part were digested in water at 20° C. for 1 hour. Starch paste was then added, and the diastase was allowed to act upon it during the following hour. A plain 2 per cent. Lintner starch paste, pH about 6.2, was used in comparison with a similar paste buffered with sodium acetate and acetic acid to a pH of about 4.5. The maltose formed by the diastase in the flour was materially increased in the more acid starch over that in the plain starch. Bran from the same wheat gave opposite results, except at lower temperatures. The results from the bran of this wheat after germination were like those from the bran of ungerminated wheat but were more pronounced.

Further investigation showed that these conditions were caused by two distinct types of diastase. The type predominating in the flour is more active in acid media, so that when the quantity of maltose produced in the more acid starch (pH about 4.5) is divided by that produced in the less acid starch (pH about 6.2) the ratio is greater than one. With the type which predominates in bran, however, the reverse is true.

Studies carried out under various conditions showed that 60° C. is an appropriate temperature to use to determine which of these two types of diastase predominate in any plant substance under investigation. Table I shows an example of the different activities found in different parts of the wheat grain at different temperatures, and with contrasting pH. It is here shown that both flour and bran contain much diastase. The relative amounts present cannot easily be stated because of differences in the conditions under

<sup>1</sup> Presented, in substance, at the meeting of the American Society of Plant Physiologists at Atlantic City, December 31, 1936.

which they give best results. The diastase in the germ is of the type found in the bran but it occurs in distinctly smaller amounts.

TABLE I  
DIASTATIC ACTIVITY IN PARTS OF AN UNGERMINATED KANSAS WHEAT\*

PARTS OF WHEAT	TEMPERATURE OF ACTIVITY				100 × RATIO B TO A
	20° C.		60° C.		
	KIND OF STARCH				
	PLAIN	BUFFERED	PLAIN (A)	BUFFERED (B)	
	pH OF SUBSTRATE				
	6.2	4.5	6.2	4.5	
	MALTOSE				
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	
Flour .....	185	280	71	148	208
Bran .....	106	122	121	68	56
Germ .....	28	28	83	23	28

\* Fifty mg. of each finely ground material digested in 5 cc. water at 20° C. for 1 hour. Added to each portion at the desired temperature, 50 cc. of 2 per cent. Lintner starch paste. Mixture digested for 1 hour. Maltose calculated to mg. in each tube.

Following the methods just mentioned, a survey has been made of the diastase in a very wide range of plant substances. The results are such as to indicate the presence of a uniform system throughout the vegetable kingdom. Determinations have been made on the carefully separated parts of newly formed and immature seeds. Diastase was nearly always found in considerable quantities. In some specimens (*e.g.*, rice) the amount was too small to be determined without the use of an inconveniently large quantity of material. In a large number of varieties of wheat and closely related cereals there was evidence of both types of diastase. In general the distribution was as in the mature and germinated grain. The one type predominated in the starchy endosperm, the other in the bran.<sup>2</sup>

Among germinated grains the two types showed clearly in wheat, barley, and rye. The results with oats, Indian corn, buckwheat, and others indicated the bran type only. Where both types of diastase are present, the sugar formed at any one point as fixed by pH and temperature is a resultant of the combined action of the two. The significance of this in determining the diastatic value of malt is apparent.

<sup>2</sup> Flour and starchy endosperm are here used interchangeably. In these separations, as in wheat milling, the aleurone layer, classed as part of the endosperm, always forms part of the bran.

Soy bean material was examined under many conditions. In all stages the flour type of diastase predominated in the cotyledons. The bran type was present in the skin. This was most readily observed in the immature beans. The bran type was also found in the pods and leaves.

Many roots, tubers, and bulbs show the bran type of diastase. A few showed the flour type to predominate. The edible sweet potato showed the flour type in a marked degree. Young shoots from the germinating sweet potato, and later the fully developed leaves, showed the bran type. The amount of diastase is very considerable in both the sweet potato and its shoots.

Leaves from many species of flowering plants selected to cover the entire range of both monocotyledons and dicotyledons showed the bran type of diastase to predominate in all of them. Taka-diastase from molds, and industrial diastase from bacteria, also showed these properties. This bran type of enzyme appears to predominate wherever active vegetation is taking place, and in most cases to such an extent as to exclude the other type. Because of this fact it has elsewhere been called *vegetative diastase*. At the same time, the type which predominates in flour and in certain other reserve material was called *reserve diastase*.

The comparison of these diastases has been extended much beyond the methods here described. In a recent series of experiments the predominating diastase in the flour of ungerminated wheat and the predominating diastase in the bran of germinated wheat were prepared in a greater degree of purity by fractional precipitation with alcohol from water extracts of the flour and bran. Solutions of these diastases in water were combined with 2 per cent. pastes from raw potato starch. Successive portions of this paste had been brought to different pH by equal quantities of sodium acetate and suitable quantities of acetic acid or decinormal sodium hydroxide.

To contrast the action of these diastases, the amounts of maltose produced at 60° C. in different portions are plotted in figure 1. Previously comparisons had been made with other and like precipitates using Lintner starch paste. In general the results were as here shown, but somewhat modified because of the partial removal of the starch coating by acid used in the preparation of that type of starch.

A study of vegetative diastase has been greatly facilitated by a liberal sample of bacterial diastase<sup>3</sup> sufficient for a long period of use. In this study, results have been obtained showing its activity at different temperatures in starch pastes of different pH and under other conditions. These studies included fractional modification or destruction of parts of the diastase by heat.

Figure 2 shows the relation of this bacterial diastase to the diastase of flour at 60° C. in pastes of varying pH. The graphs are similar to those in

<sup>3</sup> Kindly supplied by Wallerstein Laboratories, New York.

figure 1 with diastase separated from water extracts of flour from ungerminated wheat and from the bran of germinated wheat. Here the bacterial diastase, like that from the bran, gives abundant maltose in starch of the higher pH, and in a restricted amount in starch of the lower pH.

The temperature range of activity of this bacterial diastase is found to extend from about 0° C. to the local boiling point of water (97° C.) with a maximum activity between 50° and 60° C. The pH range in the more favorable temperature is from about 3.8 to 7.8. The optimum pH in the more favorable temperatures is about 5.2 to 6.2 with a tendency toward the latter

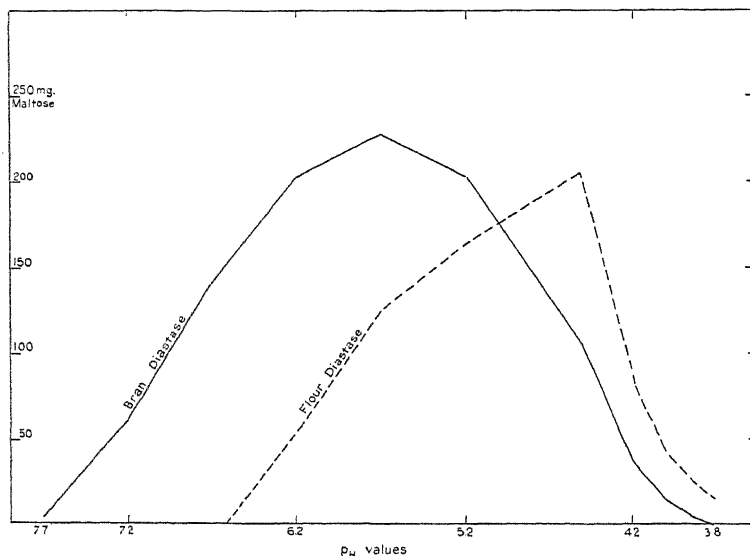


FIG. 1. Maltose formed in 60 min. at 60° C. by diastase precipitated from water extracts of bran of germinated wheat and flour of ungerminated wheat. Substrates were 50-cc. portions of 2 per cent. pastes of raw potato starch, with ten different pH values obtained by buffering with sodium acetate, acetic acid, and decinormal sodium hydroxide. Values given are as found at 25° C. Reduction was determined with potassium ferricyanide, using BLISH and SANDSTADT tables (1).

value. At about 65° C. there is a rapid falling off in the amount of sugar produced and a steady decrease in activity from this temperature upward.

The optimum conditions for the activity of liquefying diastase are so similar to those for this latter sugar-forming diastase that actual separation of the two is difficult. Their properties and differences have been compared by other means. When a suitable amount of liquefying diastase is present there is at suitable temperature and pH a rapid clearing of the opalescence of starch paste. With the bacterial diastase used, even as little as 1 mg. in water added to 50 cc. of paste containing 1 gm. of raw starch will, under

suitable conditions, become clear within one-half minute. The optimum temperature for this clearing is from 70° to 80° C. with a pH of about 6.8. In all of the many tests made under different conditions, this change has been accompanied with the production of a small amount of sugar. The liquefying diastase of germinated wheat and other cereals was found to have properties similar to those of the diastase from bacteria, but specimens from these sources seemed to be less suited to detailed study.

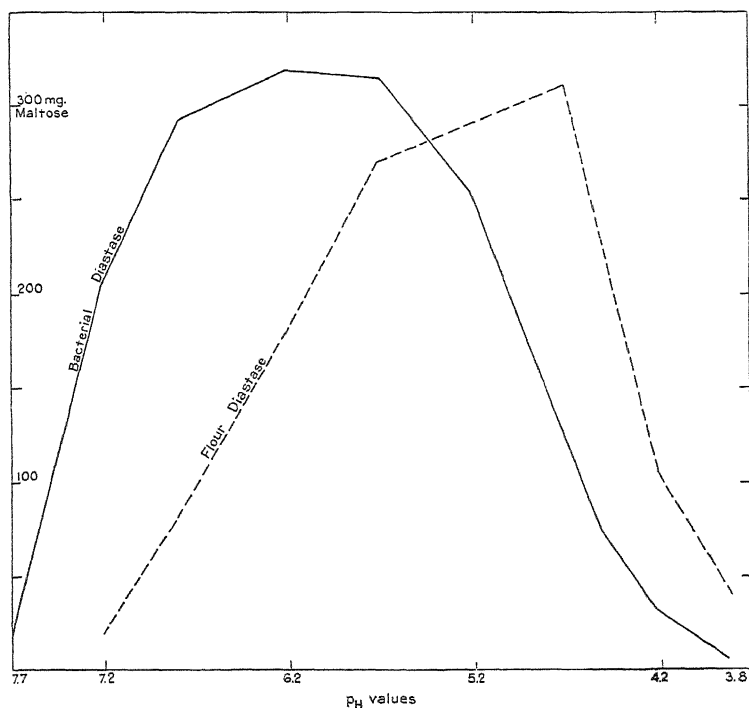


FIG. 2. Maltose formed by 1 mg. bacterial diastase and diastase in water extract from 100 mg. flour of ungerminated wheat. Other conditions as for figure 1. Note similarity of graph for bacterial diastase and for precipitated diastase from wheat bran.

With 5 mg. of bacterial diastase used in a 2 per cent. paste of 1 gm. of raw potato starch (pH 6.8), the liquids cleared in one-half minute. At six temperatures differing by 10 degrees each, the amount of reducing substance produced, calculated as maltose, did not in any case exceed 2 per cent. of the original starch. At 97° C. the clearing was complete in three-quarters of a minute. If longer time is allowed for the clearing, it will take place after the diastase has been in boiling water for several minutes. This emphasizes the resistance of the liquefying diastase to heat. When heated in water solution at 90° C. for 10 minutes, then added to 2 per cent. paste of raw potato

starch at 40° and 50° C. and the liquid held at these temperatures, the formation of sugar proceeded slowly for many days. The sugar first formed gave phenyl-hydrazine reaction for maltose. In later stages, reaction for dextrose was obtained. The clear blue test with iodine obtained in the early stages of this reaction gradually gives way to violet, red, and yellow.

Bacterial diastase was heated in water for 10 minutes at 85° and 90° C. Equivalents of 7½ mg. each were then added to several 50-cc. portions of 2 per cent. unbuffered paste from raw potato starch, pH about 6.8, and kept at 50° C. In three hours, 95 per cent. and 90 per cent. of the opalescence had disappeared with reduction equivalent to 7 and 5 mg. of maltose respectively. At 20 hours, solutions were completely clear, with reduction equal to 55 and 30 mg. of maltose. Reduction steadily increased, and at 312 hours showed an equivalent of 415 and 385 mg. of maltose.

Figure 3 shows graphs of sugar production with 1 mg. of unheated diastase in comparison with 50 mg. of the diastase heated at 80° and 85° C.

TABLE II

ACTIVITIES OF BACTERIAL DIASTASE AFTER HEATING IN WATER AT 95° C.\*

DIASTASE MINUTES HEATED	TEMPERA- TURE OF DIGESTION	HOURS DIGESTED					
		40 HOURS			64 HOURS		
		MALTOSE	CONDI- TION OF LIQUID	IODINE REACTION	MALTOSE	CONDI- TION OF LIQUID	IODINE REACTION
<i>min.</i>	<i>°C.</i>	<i>mg.</i>			<i>mg.</i>		
5	40	40	Clear	Blue	58	Clear	Blue
8	40	31	Faint Cloud	Blue	44	Clear	Blue
11	40	22	Faint Cloud	Blue	30	Clear	Blue
5	50	60	Clear	Blue	87	Clear	Violet
8	50	51	Clear	Blue	96	Clear	Violet Blue
11	50	23	Faint Cloud	Blue	33	Clear	Blue

\* Three lots of 250 mg. bacterial diastase heated in 100 cc. water for 5, 8, and 11 minutes, cooled and 3-cc. portions added to 50 cc. of 2 per cent. paste from untreated potato starch. This is equivalent to 7½ mg. of diastase to 1 gm. of starch. One series was digested at 40°C. the other at 50° C. Solutions were examined at intervals. Examples given show the amounts of maltose formed in 40 and 64 hours, with other observational data.



for 10 minutes. The pH value of the substrate was about 5.2 at 25° C. but it changes consistently with the temperature. The change in relative positions of the graph lines with these changes is significant. Near 65° C. they cross each other. The sugar formed by 50 mg. of the liquefying diastase which has survived heat for 10 minutes at 80° C. is now distinctly higher than from the smaller amount of liquefying diastase in the 1 mg. of unheated material.

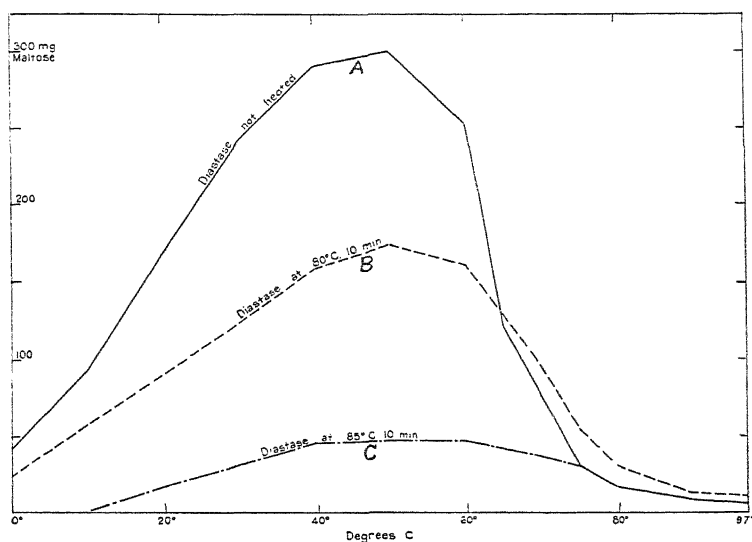


FIG. 3. Maltose formed in 60 min. at different temperatures in 50-cc. samples of 2 per cent. raw potato starch paste, pH 5.2 at 25° C. A = 1 mg. diastase not heated. B = 50 mg. diastase from 1 gm. heated in 100 cc. water at 80° C. for 10 min. C = 50 mg. diastase from 1 gm. heated in 100 cc. water at 85° C. for 10 minutes. Boiling point of water was 97° C.

On the other hand the 1 mg. of unheated diastase produced a very much larger proportion of sugar below the critical temperature of 65° to 70° C. than was produced by 50 mg. of the heated diastase. Similar changes were found in all of a large number of determinations under different conditions. With 50 mg. of diastase heated to 85° C. the sugar formation at the higher temperatures still equals that from the 1 mg. of unheated diastase, but in this case there is much less sugar formation at the lower temperatures than with either of the other portions. These data, in connection with other information, indicate the presence of two types of diastase in this group: a predominantly sugar forming type and a predominantly liquefying type.

The problem of the starch granule, its structure, and its formation is intimately associated with the problem of diastase. A study of the diastases sheds new light on both problems. By use of the diastases the obscurity which surrounds the formation of starch granules has been removed and

something of what lies beneath has been observed. Figure 4 shows a series of stages in the breaking down of natural, uncooked potato starch under the slow action of bacterial diastase; figure 5 shows potato starch in formation; and figure 6 shows some instructive miscellaneous potato starch formations. Numerous photographs were made of selected specimens as they lay scattered among others under the microscope. For repeated examinations, the micro-

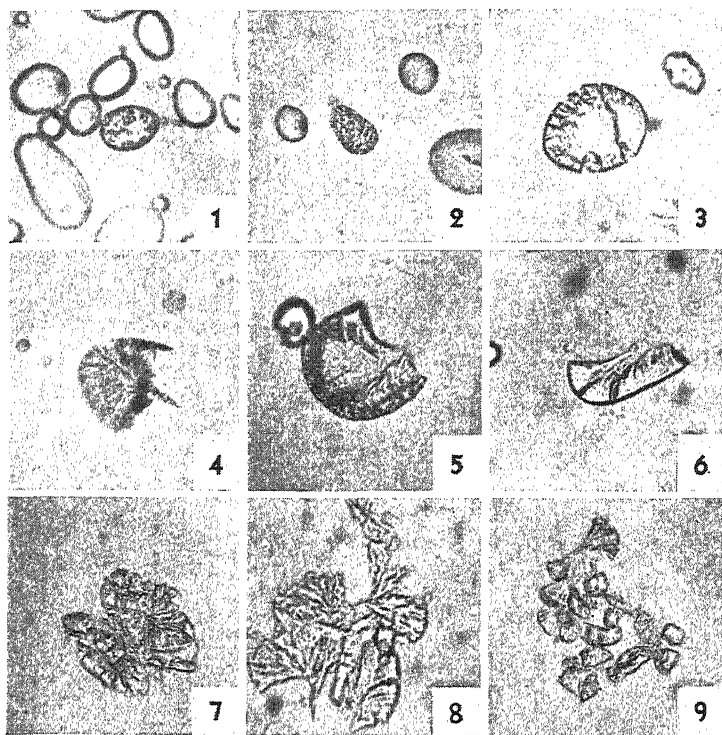


FIG. 4. Potato starch disintegrated by bacterial diastase. 1, coating partly removed. 2, coating completely removed. 3, granule much swollen showing break at right angles to longer axis. 4, group of segments forming long point. 5, segments loosened and spread. 6, smaller group showing long segments. 7, enlarged granule showing loosened segments closely grouped. 8, segments of tree-like formation. 9, smaller segments.

scopic slides with the starch in the solutions of diastase were kept moist in a covered petri dish at a temperature somewhat below the gelatinizing point of the starch. Vegetative diastase, whether obtained from bacteria, from germinating seeds, or from other materials, is similar in its action.

The numbers in parentheses in the following paragraphs correspond to the numbers (1 to 27) in figures 4 to 6. At first the outer coating of the starch grain is eaten away in patches (1). When this is completely removed,

the remainder of the granule presents a surface of jagged points suggesting an Osage orange (2). Gradually the granule becomes enlarged from the absorption of liquid (3). In many cases numerous spaces are seen between the points, leaving a deeply serrated edge. Frequently a crack appears across this enlarged granule and at right angles to the main axis. The parts

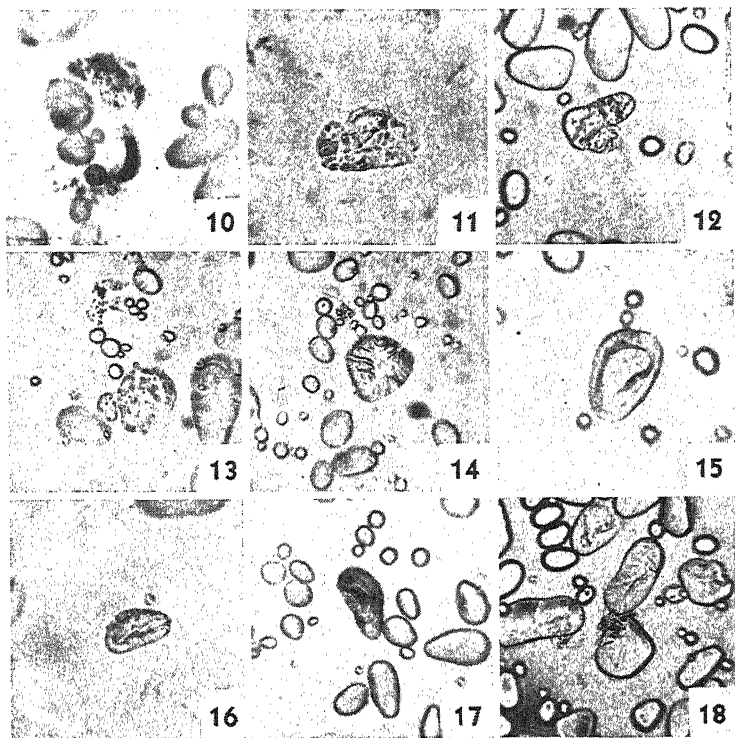


FIG. 5. Potato starch in formation. 10, wisps of loose starch substance lightly stained with iodine. 11, more concentrated aggregate of starch substance. 12, another aggregate with slight coating at bend. 13, circular granule with light coating. 14, group of aggregates with light coating. 15, well-shaped granule with coat forming from border toward center. 16, coating extending from border toward center. 17, coating more nearly complete; granule more coated and blued with iodine. 18, group of completed and nearly completed granules, one at center with new starch aggregate near end.

separate. One shows a long point (4, 23, 24), the other a funnel-shaped opening from which the point was withdrawn (24).

As these parts disintegrate they are seen to have been made of series of small segments. Some are long, straight, and narrow (6). Others are bent, with the broader end abutting the surface at nearly right angles (5). The narrow end of these is directed toward the so-called point of growth and the tip of the long projection first mentioned (4) appears to stop at that point.

Those segments from the end nearest the point of growth are short, showing triangular or wedgeshaped outlines (9). Sometimes the segments show a treelike formation (8). These may separate and become much enlarged as by the absorption or imbibition of water. Many segments are seen with channels through them, and appear to be tubular in form. This is sometimes due to spaces between two or more of them, but even very minute single seg-

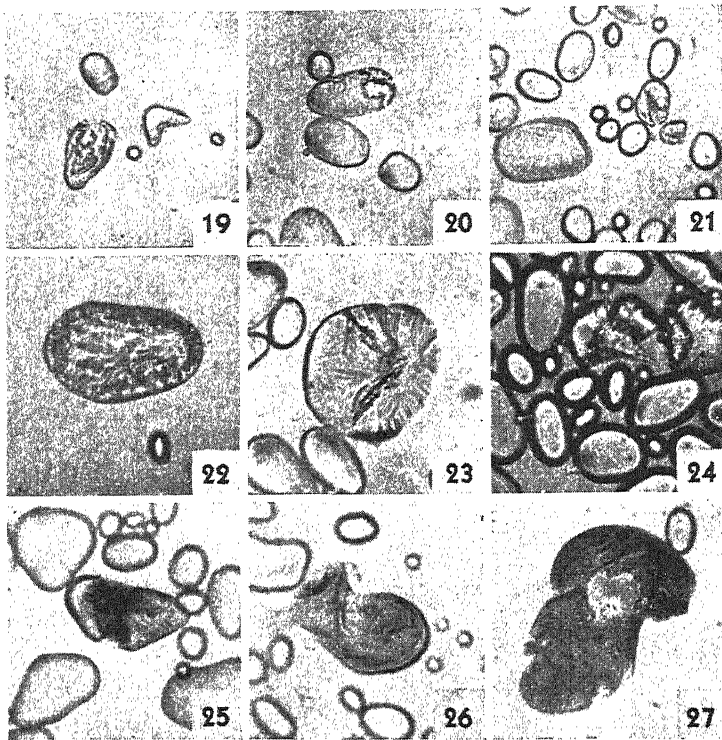


FIG. 6. Miscellaneous potato starch formations. Nos. 19, 20, 21, granules with interior partly digested from within the incomplete coatings. 22, swollen granule without coatings. 23, parted granule as in 24 with point upward. 24, parted granule showing point and pit from which it was withdrawn. 25, granules with new starch formation between them showing loosened coatings with ends bent back. 26, 27, monster granules without coatings.

ments show this apparent tubular construction. It was first noticed under the slow action of diastase from germinated Indian corn. These various fragments are blued with iodine. With very dilute iodine they do not show solid color, but are blued in parts as if made of more than one substance.

It is commonly accepted that enzymes not only break down, but, under other conditions, build up the food reserves. In the young growing potato we see something of how starch granules are formed. Thin, hazy, irregular

aggregates of matter appear as starch on staining slightly with very dilute iodine (10). Various others are becoming gradually more dense (11, 12). Still others are seen partly covered with a thin coating (13, 14). The ragged outline of the irregular aggregates is being subdued and becomes shaped more nearly in the form of a granule. Sometimes irregular portions of considerable size extend from the main portion (12). Others are seen with a heavier coating showing on the surface, the characteristic appearance of a typical granule, but disconnected in places (15, 16, 17).

These incomplete granules can readily be seen scattered among many granules by adding a little dilute iodine. This stains the exposed interior a deep blue while the coating and grains fully coated are little affected. The antecedents of the granules, the appearance of the exposed interior of the incomplete granules, and the results of the action of diastase on them show clearly their lack of organization. When diastase acts on the interior it is eaten away, leaving the coating as a partial shell (19, 20, 21, and many more perfect specimens not here shown). In these imperfect granules we do not obtain the same characteristic segmentations by the continued action of diastase as is found when it acts on mature granules.

The enlargement of granules by deposit of starch aggregates on the surface is frequently seen (18). These become coated as was the original granule and thus become a part of it. Frequently during this process the coating is seen to have been dissolved and the loose ends spread or folded back (25). Numerous large granules show clearly from their outlines that they have been enlarged in this way.

That this general method of starch formation is not confined to the potato is apparent from a similar but less complete study on frosted wheat. Here the kernels are still green from being stopped in the midst of their development. The partially formed granules are somewhat as described for the potato. Photographs of various starches seen in the extensive work of REICHERT (2) give evidence of such formation of the starch of other plants. However, the starches used for those several hundred photographs had been separated and purified by washing in water (part I, p. 299). This must necessarily remove a considerable part of the partially formed granules. This volume contains much excellent information concerning the previous literature on starch.

Some unusual photographs are seen in figure 6. In one new potato several large swollen granules were noted. They had thin coats over them. Under the influence of bacterial diastase one was seen gradually to loosen this coat. It then formed a crack and separated into parts leaving the usual long point on the one part and the pit in the other. In various instances the point of a separated granule was directed upward (23). The general uniformity of the outlines of the two parts of a separated granule are shown in

figure 6 (24). The enlarging of a starch granule by formation of an aggregate of starch substance between two granules was occasionally seen (25). Here a part of the coating on each has folded back to permit the inclusion of the new aggregate. In one new potato several uncoated monster granules were seen (26, 27). In dilute iodine these stood out as an intense blue mass. That no coating surrounded them was clearly apparent. This emphasizes the point that in the formation of the starch granule, as in its dissolution, two active agents take part.

In a review of these observations in early April new potatoes, so immature that the skin has peeled freely in shipping, showed many starch granules in the process of formation as described. In several other potatoes, new but more mature, the newly formed granules were rare. This is construed as further confirming the usual sequence in formation of starch as here described.

During the present summer (1937) opportunities have been found for a further study of young and growing specimens of starch-producing substances. These have included certain cereals, tubers of the spring beauty, and other early flowering plants, besides acorns, thus representing widely different families. Some tubers of the Colorado wild potato (*Solanum jamesii*) have also been useful. In the many observations made, there was abundant evidence in all these specimens of conditions similar to those in new potatoes.

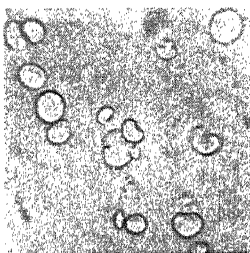


FIG. 7. Starch from young spelt.

In spelt, wheat, barley, and rye, seeds were taken shortly after blossoming and later. At first very minute starch granules were seen. Soon larger crescent shaped granules appeared. From the inner side of many of these a projection was seen. They then showed an outline similar to that of a section through the center of the ordinary cultivated mushroom and parallel to the stem. Gradually there were larger granules and the crescent became filled much as the moon fills during the second quarter. During this stage the inner portion is often seen to be imperfectly coated while the outer curve shows a distinct coating. These forms are seen in figure 7. In many of these seeds, especially in rye, loose aggregates of coagulated starch substance

are seen. These appear as loosely constructed flat discs, such as when coated would form flat or lenticular starch granules like those common to these cereals.

The specimens of Colorado potato tubers examined showed many uncompleted granules and many partly coated aggregates of starch substance. When an active diastase acts upon these imperfect granules the interior is often eaten away leaving portions of overhanging starch coating. This has been seen when using the diastase of malt, molds, bacteria, the saliva, and the pancreas. The ragged overhanging starch coating seen in this way shows a marked contrast to the well-rounded edges of the newly formed coating as seen in slides prepared without the added diastase. These contrasts may be seen in the upper row of figure 6 in comparison with specimens shown in figure 5.

### Summary

1. The grains of certain cereals, and parts of some other plants, contain two types of sugar-forming diastase. Both are markedly influenced by changes in temperature and pH but in opposite directions, especially at the higher temperatures of their activity. The one predominates in the flour of wheat, the other in the bran, although both are present in each. The optimum pH value for the former is about 4.5, and for the latter about 5.3 and above.

2. That type of diastase which predominates in wheat bran is widely distributed in the vegetable kingdom. It is most abundant where active vegetation is taking place, and, with the liquefying type, may be called *vegetative diastase*. These two are the types which develop most prominently during germination. That type which predominates in flour is limited in its distribution. It is present in certain substances containing much reserve starch and may be called *reserve diastase*.

3. Closely associated with the vegetative sugar-forming diastase is another which is predominantly liquefying in its properties. Under suitable conditions it will clarify starch paste with great promptness leaving a water-clear solution. Immediately after liquefying, this solution gives a clear blue with iodine and may contain only very small quantities of reducing substance. Under other conditions liquefying diastase removes the outer coating from starch granules and exposes the interior to the action of the other diastases.

4. By a careful study of young growing potatoes, starch granules are seen in various stages of development. There is first a thin amorphous haze which is blued by very dilute iodine. This is followed by successive stages of more dense aggregations. Around each a coating forms. This binds the interior into the completed granule and protects it against solution. Granules are often enlarged by deposits of starch substance at one or more points on the

surface. The coating is extended to cover this and the whole becomes part of the enlarged granule. Evidence of similar starch formation is seen in developing wheat grains and elsewhere.

5. The interior of the granule, as first formed, is amorphous. Later, and apparently only underneath the coating, it becomes organized into series of segments. When the coating is carefully removed as by liquefying diastase, groups of segments, and eventually many segments, are gradually released. These appear as various regular forms, often of geometric outlines; sometimes funnel shaped, sometimes arborescent. Acted on further by diastase they diminish in size, then pass wholly into solution and we find soluble starch, dextrin and, or sugar in their stead.

The author is greatly indebted to Dr. FRITZ LAUN of the Columbus Laboratories for his patience and painstaking efforts in photographing the specimens selected to illustrate points here given.

THE COLUMBUS LABORATORIES  
CHICAGO, ILLINOIS

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# RESPIRATORY BLOCK IN THE DORMANT SPORES OF *NEUROSPORA TETRASPERMA*

DAVID R. GODDARD AND PAUL E. SMITH

(WITH FIVE FIGURES)

## Introduction

SHEAR AND DODGE (26) have shown that the ascospores of *Neurospora tetrasperma* are dormant, but that they may be induced to germinate by a short period of heating. This process of heat activation was studied by GODDARD (7), who found that heating the dormant spores to temperatures of over 50° C. for a few minutes induced germination two to three hours after returning them to room temperature. Further, he found that the activation is reversible. If the respiration of the activated spores was prevented for several hours by anaerobic conditions or the addition of cyanide, upon returning to conditions favorable for respiration, the spores failed to germinate; that is, they had been de-activated. If the de-activated spores were re-activated by a second heat treatment, they germinated normally. The activation of the spores induced a large increase in the respiratory rate (8 to 40 times), and this high rate of respiration had to continue for two to three hours if germination was to occur. Upon germination, a second increase in the respiratory rate was found, nearly doubling that of the activated spores. Thus, he recognized three phases in the rate of respiration of the ascospores: (1) that of dormant spores, (2) that of activated spores (one-half hour to two hours after the heat treatment), and (3) that of germinating spores (the spores in which germ tubes may be seen under the microscope).

In this paper we have tried to determine what constitutes the respiratory block of dormant ascospores of *Neurospora tetrasperma*, or, conversely, what part of the respiratory mechanism undergoes heat activation. It is to be realized that activation and germination occur readily in distilled water, so that an external substrate is not essential for activation or germination. We may assume concerning the respiratory systems of dormant and activated spores, either (a) that the dormant and activated respiratory mechanisms are qualitatively dissimilar or even independent, and that heat treatment causes a *de novo* appearance of a system inactive in dormant spores, or (b) that the same mechanism is functional in dormant and activated spores, but that in dormant spores the rate is limited by the slowest process in the total reaction chain, and that this slow reaction is greatly accelerated by the heat treatment, allowing the total respiratory chain to proceed at the rate found in activated spores. If the first assumption is correct, the respiratory block is the inactivity of the reaction which prevents the second

respiratory mechanism from functioning, while if the second assumption is correct, the limited activity of a certain reaction constitutes the respiratory block.

We have accepted the WARBURG-KEILIN scheme of cellular respiration as a working theory, and we have summarized this scheme in abbreviated form in diagram I. For reviews of this theory, see KEILIN (10, 11) and

### DIAGRAM I

#### PHAEOHEMIN—CYTOCHROME—DEHYDROGENASE THEORY

- (1) Reduced phaeohemin +  $O_2 \xrightarrow[\text{by CO}]{\text{Poisoned}}$  Oxidized phaeohemin
- (2) Oxidized phaeohemin + Reduced cytochromes  $\xrightarrow[\text{by HCN}]{\text{Poisoned}}$  Reduced phaeohemin + Oxidized cytochromes
- (3) Oxidized cytochromes + Reduced dehydrogenases  $\longrightarrow$  Reduced cytochromes + Oxidized dehydrogenases +  $H_2O$
- (4) Reduced substrate + Oxidized dehydrogenase  $\longrightarrow$  Oxidized substrate + Reduced dehydrogenase

Oxidized substrate may undergo reaction with a second dehydrogenase, or it may undergo decarboxylation and then oxidation. Flavine enzyme, coenzymes, etc., omitted. Either (3) or (4) inhibited by urethanes.

MELDRUM (17). Certain workers have long believed that the first reactions in respiration are identical with the first steps of alcoholic fermentation. The older literature supporting this opinion is reviewed by KOSTYCHEV

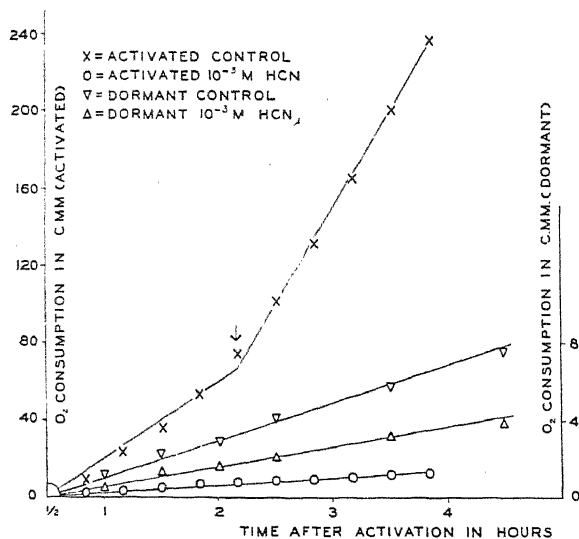
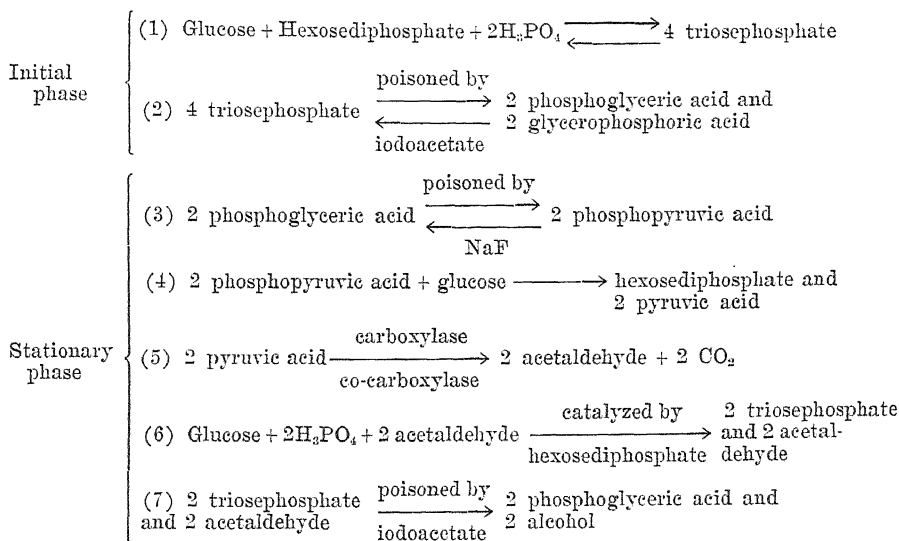


Fig. 1. The effect of 0.001 M. HCN on the respiration of dormant and activated ascospores, 6.48 mg. dry weight of spores per vessel. The arrow indicates the beginning of germination.

(13) and, more recently, by TURNER (32). Since in the work which follows we have had to discuss certain reactions which are usually considered as part of the mechanism of alcoholic fermentation, we have summarized the MEYERHOF-KIESSLING theory of alcoholic fermentation in diagram II. For a review of this theory, see MEYERHOF (19) and MEYERHOF AND KIESSLING (22).

## DIAGRAM II

### MEYERHOF-KIESSLING SCHEME OF ALCOHOLIC FERMENTATION



## Methods

The *Neurospora tetrasperma* culture was the same strain used in the earlier work (7), and the same method of collecting and separating the ascospores was followed. The cultures were grown at 25° C. for three to four weeks in 500-ml. Blake bottles on 35 to 50 ml. of agar 0.5 per cent. for glucose and Difco malt extract. The spores were stored at constant relative humidity over saturated  $\text{NH}_4\text{Cl}$ /solid  $\text{NH}_4\text{Cl}$ . In the earlier experiments the spores were stored at room temperature, but in the later experiments at about 4° C. Spores stored at room temperature gave fairly uniform results for several weeks; those stored at 4° C. have given very uniform results over several months. In the experiments reported here, the spores from more than four thousand cultures were used, and we wish to thank Miss EUGENIA SHERIDAN who has carried out much of the laborious culture work.

All measurements were made with the FENN (6) volumetric micro-respirometer, at 25° C., unless stated to be at 15° C. This instrument is a

closed system, and is very satisfactory for the low rates of dormant spores. The rate of shaking was 180 complete cycles per minute, through an arc of 1.5 cm. A small spherical glass bead was included in each vessel to enhance stirring. The high density (about 1.18) of the spores caused some shaking difficulties, but in none of these experiments was the rate of shaking limiting. Carbon dioxide was absorbed in KOH insets, unless respiration was measured in the presence of carbon dioxide; in this case, WARBURG's indirect method (5) was used. In the HCN experiments (KREBS, 14), KOH KCN mixtures were used in the insets. The spores were suspended in M/60 phosphate buffers at a pH of 5.4 or in distilled water. The substrates and poisons were neutralized and made up in the same buffer. The poisons or substrates were added to the vessels at the beginning of an experiment, with other vessels serving as controls. All experiments were run in duplicate, and the experiments reported are only a few of many which gave similar results. The volume of cells used in an experiment was determined by centrifuging to constant volume in hematocrit tubes. Very uniform results may be obtained with *Neurospora spores* by this means. In a few cases, both dry weight and hematocrit determinations were made on the same suspension, and it was found that the volume multiplied by 0.36 equals the dry weight. In most experiments, the  $-Q_{O_2}$  was calculated from volume determinations by the use of this factor.

The spores as collected were dormant. Activation was accomplished by heating the spore suspension in a test tube for twenty minutes at 54–56° C. in a water bath, and then cooling to room temperature. Zero was taken as the time at which the spores were removed from the 54° C. bath.

Pure nitrogen for anaerobic experiments was obtained by passing tank nitrogen over freshly reduced copper in an electric furnace. A small piece of yellow phosphorus was used in the inset to absorb any oxygen not swept out of the vessel with nitrogen. Carbon monoxide was prepared by the dehydration of concentrated formic acid with hot sulphuric acid, and was washed with KOH. Gas mixtures were made by volume, and were not checked by analysis.

The hexosediphosphate was a gift from the Winthrop Chemical Company. It was converted to the sodium salt, decolorized with charcoal, and re-precipitated as the calcium salt with alcohol. The calcium salt of the hexosediphosphate was converted to the sodium salt by adding the theoretical amount of sodium oxalate and filtering. The solution of sodium hexosediphosphate was neutralized, when necessary, with HCl. The methyl glyoxal was prepared by M. P. SCHUBERT of the Rockefeller Institute. The pyruvic acid was made by the method of HOWARD and FRASER (9), and was distilled at low pressure.

## Results

## THE RESPIRATORY INCREASE ON ACTIVATION AND GERMINATION

A few typical results are listed in table I. The respiratory rate of activated spores varies from about 8 to 40 times that of the dormant spores,

TABLE I  
RESPIRATION OF DORMANT, ACTIVATED, AND GERMINATING SPORES  
OXYGEN CONSUMPTION AS  $-Q_{O_2}$ \*

SPORE LOT	SPORE AGE	DORMANT	ACTIVATED 1-1.5 HR. AFTER ACTIVATION	GERMINATION 3-5 HR. AFTER ACTIVATION
A .....	<i>wk.</i> 3	0.25, 0.27	10.86	19.45
A .....	9	0.53, 0.59	4.48	9.62
B .....	3	0.25, 0.24, 0.27	10.36	19.60

$$* - Q_{O_2} = \frac{\text{cu.mm. oxygen consumed per hr.}^1}{\text{dry wt. in mg.}}$$

<sup>1</sup> The symbols  $Q_{O_2}$ ,  $Q_{CO_2}$ , etc. are by convention negative when the gas is consumed and positive when it is produced.

depending upon the age of the spores. The  $-Q_{O_2}$  of dormant spores increases from about 0.25 when first collected to 0.50-0.70 when several weeks older, while the  $-Q_{O_2}$  of activated spores decreases from about 10 when first collected to about 5-6 several weeks later. If the spores are stored at 4° C. the values remain practically constant at 0.5 and 5 to 6 respectively for several months. When the spores have been stored for 3 to 4 months at room temperature the  $-Q_{O_2}$  values of dormant and activated spores have decreased to about 0.08 and 1 to 2 respectively, and only a few per cent. of the spores germinate after a heat treatment.

Approximately two hours after activation, if respiration has been allowed to proceed normally at 25° C., and four hours, if at 15° C., a second change in respiratory rate occurs, and this corresponds with the first appearance of the germ tubes. The respiratory rate of germinating spores also varies with the age of the spores, but in such a manner that the absolute value is about twice that of the activated rate. The average value from ten experiments with spores of various ages was 1.98 times the activated rate, and the range was from 1.79 to 2.15. The number of spores germinating seemed to be quite independent of the rate of oxygen consumption as long as the  $-Q_{O_2}$  for activated spores was 4 to 5 or higher.

## EFFECT OF OXYGEN AND CARBON DIOXIDE PRESSURE ON RESPIRATION

It is possible that the permeability of the dormant spores to carbon dioxide or oxygen limits the respiratory rate. If permeability to oxygen

TABLE II  
EFFECT OF O<sub>2</sub> AND CO<sub>2</sub> PRESSURE ON DORMANT SPORES  
OXYGEN CONSUMPTION AS - Q<sub>O<sub>2</sub></sub>

GAS PRESSURE	OXYGEN*			CO <sub>2</sub> †	
	A	B	C	D	E
<i>mm. Hg.</i>					
0 .....	—	—	—	0.33	0.71
37 .....	0.15	—	—	0.30	0.65
75 .....	0.15	—	0.66	0.37	0.66
150 .....	0.17	0.27	0.65	0.30	—
190 .....	—	—	—	—	0.58
300 .....	0.16	0.26	—	—	—
750 .....	—	—	0.77	—	—

\* CO<sub>2</sub> pressure = zero.

† O<sub>2</sub> pressure = 150 mm.

were limiting, we would expect an increase in the respiratory rate by raising the partial pressure of oxygen. The data in table II show that raising the oxygen pressure from 37 to 300 mm. has practically no effect on the rate. If the retention of CO<sub>2</sub> by the spores were limiting, it would be expected that increased pressures of CO<sub>2</sub> would decrease the rate. The data in table II show that this is not the case. The results reported in table III

TABLE III  
EFFECT OF OXYGEN TENSION ON ACTIVATED AND GERMINATING SPORES  
OXYGEN CONSUMPTION AS - Q<sub>O<sub>2</sub></sub>

OXYGEN PRESSURE	A 25° C.		B 25° C.		C 15° C.	
	ACTIVATED	GERMINATING	ACTIVATED	GERMINATING	ACTIVATED	GERMINATING
<i>mm. Hg.</i>						
75 .....	—	—	6.04	6.90	3.07	7.55
150 .....	6.94	10.17	7.91	15.65	3.15	7.03
300 .....	—	—	8.00	16.60	3.23	—
450 .....	8.90	11.40*	—	—	—	—
750 .....	8.92	10.25*	—	—	—	—

\* High partial pressures prevent normal germination.

show the effect of oxygen tension on germinating and activated spores. At oxygen tensions below 20 per cent., the oxygen diffusion rate may be limiting the rate of respiration in activated and germinating spores at 25° C. but not at 15° C., where the absolute rate is lower. It seems very improbable that the permeability of the dormant spores to CO<sub>2</sub> or oxygen governs their rate of oxygen consumption.

## THE PHAEOHEMIN-CYTOCHROME SYSTEM

The work of WARBURG and KEILIN, which is briefly summarized in diagram I indicates that the cyanide-carbon monoxide sensitive respiration of most cells is catalyzed by the phaeohemin-cytochrome system. From diagram I it can be seen that both cyanide and carbon monoxide poison the phaeohemin enzyme. In an attempt to determine whether the rate of respiration of dormant spores was limited by the activity of the phaeohemin enzyme, we have studied the effect of cyanide and carbon monoxide on the respiration of dormant and activated spores. The results are shown in figures 1 and 2 and in tables IV and V. It is apparent from these results

TABLE IV  
PERCENTAGE INHIBITION OF RESPIRATION OF SPORES BY HCN

HCN CONCENTRATION	DORMANT	ACTIVATED	GERMINATING
$1.0 \times 10^{-4}$ Molar .....	7.2; 5.4; -7.5	41.6; 23.2	55.6; 53.4
$1.0 \times 10^{-3}$ Molar .....	49.2; 45.3	86.8; 86.8	94.4; 94.1†
$1.0 \times 10^{-2}$ Molar .....	35.5*	87.5	95.6†

\* Result not very reproducible; HCN poisoning is not reversible at  $1.0 \times 10^{-2}$  molar. Lower concentration always reversible.  $1.0 \times 10^{-2}$  molar sometimes stimulates dormant spores slightly, while  $1.0 \times 10^{-3}$  molar always inhibits.

† Controls germinating; HCN prevents poisoned spores from germination.

TABLE V  
PERCENTAGE INHIBITION OF RESPIRATION OF SPORES BY CARBON MONOXIDE\*

GAS MIXTURE	DORMANT	ACTIVATED	GERMINATING
90% CO†/10% O <sub>2</sub> .....	5.6; -5.0; 7.7; 15.4	53.3; 43.4; 50.2; 31.6; 38.3; 42.3	54.7; 17.1; 26.0; 8.8
95% CO‡/5% O <sub>2</sub> .....	21.4; -6.0	73.3	—
Av. ....	5.7	43.15	23.64

\* In the dark.

† Controls 90% N<sub>2</sub>; 10% O<sub>2</sub>.

‡ Controls 95% N<sub>2</sub>; 5% O<sub>2</sub>; these results at 15° C.

that the respiration of the activated spores is much more sensitive to these poisons than is the respiration of the dormant spores. Not only are the dormant spores less sensitive to these poisons, but about 50 per cent. of the respiration is entirely resistant to cyanide. A comparison of the absolute rates of respiration of cyanide poisoned dormant and activated spores is of interest. The results from one experiment gave the following: the  $-Q_{O_2}$  values for dormant spores and dormant spores plus  $10^{-3}$  and  $10^{-2}$  molar HCN were 0.27, 0.14, and 0.17 respectively. The corresponding values for

activated spores were 10.86, 0.65, and 0.30. These  $-Q_{O_2}$  values show that while most of the activated respiration is poisoned by HCN, cyanide does not bring the respiration quite as low as that of cyanide poisoned dormant spores. Cyanide at  $10^{-3}$  and  $10^{-2}$  molar inhibits 93.5 and 97.1 per cent. of the increased respiration due to activation (the increased respiration equals

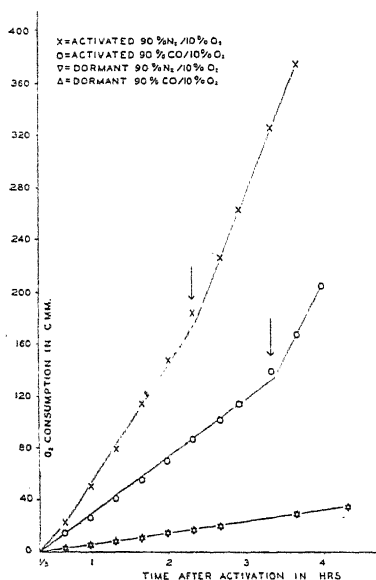


FIG. 2. The effect of carbon monoxide on the respiration of dormant and germinating ascospores, 7.95 mg. of spores per vessel in the activated experiment and 14.90 mg. of spores per vessel in the dormant experiment. The arrows indicate the beginning of germination.

the activated respiration minus the dormant respiration). The cyanide resistant respiration may be catalyzed by WARBURG's flavine enzyme since it is known (30) that the enzyme is not cyanide sensitive. However, it is clear that 93 to 97 per cent. of the increased respiration due to activation is cyanide sensitive, so that the activity of the flavine enzyme (if it is present) cannot account for the increased respiration on activation. The carbon monoxide results show that the dormant respiration is almost completely carbon monoxide resistant, while the activated respiration is definitely carbon monoxide sensitive.

If the rate of respiration of the dormant spores is limited by the concentration of active phaeohemin, the dormant respiration should be as sensitive to cyanide and carbon monoxide as is the respiration of the activated cells. However, if the concentration of the phaeohemin is as high in the dormant spores as in the activated spores, only 1/8 to 1/40 of the enzyme is essential to maintain the observed rate, and poisoning a large percentage



of the enzyme with carbon monoxide or cyanide will have little or no effect on the dormant rate, while a large inhibition of the activated rate may be expected. This is what has been found experimentally. Cyanide at  $10^{-4}$  molar should combine with about 50 per cent. of the phaeohemin, but it has no effect on the dormant respiratory rate. Higher concentrations, as  $10^{-3}$  molar, will combine with 95 per cent. or more of the enzyme. The affinity of carbon monoxide for phaeohemin is much less than is that of cyanide, and it may be impossible, at pressures of atmospheric or less, to tie up sufficient phaeohemin in dormant cells to produce an appreciable inhibition. Following this line of argument, we have interpreted our cyanide and carbon monoxide results to mean that the respiratory rate of the dormant spores is not determined by phaeohemin activity.

In the WARBURG-KEILIN scheme, cytochrome is believed to act as a carrier between phaeohemin and the dehydrogenases. No specific cytochrome poison is known. Cytochrome activity may be followed spectroscopically in some cases, but the black spore pigment has made that impossible in this work. One method of attack on phaeohemin-cytochrome systems is the one introduced by BARRON (2), and used by many workers, of "short circuiting" both the phaeohemin oxidase and cytochrome by the addition of autooxidizable dyes (such as methylene blue, pyocyanine, and thionine) to the cell suspensions. These dyes are reduced by dehydrogenase-substrate systems and re-oxidized directly by atmospheric oxygen, and therefore these dyes may replace both oxidase and cytochrome. Another dye which RUNNSTROM (25) has used in paraphenylenediamine. This dye is not autooxidizable. It is reduced by the dehydrogenase-substrate systems, and its oxidation is catalyzed by phaeohemin. This dye will supplement cytochrome, but not phaeohemin. We have added such dyes to dormant spores, and no significant change in the respiratory rate has been detected, as is seen in table VI. These results may be interpreted to mean that the respiratory block is not

TABLE VI  
EFFECT OF DYES ON DORMANT SPORES  
OXYGEN CONSUMPTION AS  $Q_{O_2}$

DYE	CONTROL	EXPERIMENTAL
Methylene blue 0.01% .....	0.23	0.31
Pyocyanine M/5000 .....	0.31	0.24
Pyocyanine M/10,000 .....	0.31	0.31
Paraphenylenediamine M/1000 .....	0.21	0.28*
Paraphenylenediamine 10 mg. per vessel .....	0.37	0.46†

\* Not corrected for autooxidation of the dye.

† Corrected for autooxidation of the dye.

in the phaeohemin-cytochrome system, or that the spores are impermeable to the dye, and that no conclusions can be drawn from these experiments.

To prove definitely that the oxidase is phaeohemin, light reversal of carbon monoxide poisoning should be demonstrated. Attempts to obtain light reversal of carbon monoxide poisoning were largely unsuccessful, probably because of the black pigment in the spore walls. The results all show slightly less inhibition by carbon monoxide in the light (7 cm. from 100-watt Mazda lamps) than in the dark, but the differences were hardly larger than the experimental error and are not reported here.

Paraphenylenediamine may be used to measure the activity of phaeohemin, by the method of KEILIN (10). In this method, a large amount of dye (10 to 23 mg.) is added to the cell suspension, and the oxygen used in oxidation of the dye is measured. Proper controls are used to correct for the autoxidation of the dye and for the cellular respiration (or this may be poisoned with urethane, etc.). The rate of the catalyzed oxidation of the dye is a measure of phaeohemin activity. Such an experiment was tried with dormant spores with negative results. This may mean that there is no phaeohemin activity (which would conflict with our CO and HCN results), or, more likely, that the cells are impermeable to the dye.

#### DEHYDROGENASE-SUBSTRATE SYSTEMS

It seems to be clearly established from the work of KEILIN (10) and others that the respiratory substrates do not react directly with oxidized cytochrome, but that the substrates first react with intermediary enzymes, the dehydrogenases of THUNBERG (31). The usual method of determining dehydrogenase activity is the methylene blue technic of THUNBERG. An attempt to use this method with *Neurospora* spores failed because the black spores removed all of the dye from solution, and the spore color made it impossible to determine whether the dye was reduced or not. (An attempt to determine this spectroscopically also failed.) The dye may not be extracted by centrifuging with water, though it can be extracted with acetone.

We know no truly specific dehydrogenase poison. Though SVENSSON (29) has shown that urethane will poison dehydrogenases, other enzymes are also poisoned by urethane. We have studied the effect of ethyl urethane on the respiration of dormant and activated spores, and a typical experiment is shown in figure 3. A higher concentration of urethane (5 per cent.) caused at first a marked decrease in respiration of dormant spores, followed by stimulation. The stimulation is probably associated with irreversible injury. If the percentage inhibition of respiration by 2.5 per cent. urethane is calculated from the data in figure 3 for dormant, activated, and germinating spores, the following results are obtained: 48, 19, and 62 per cent.

respectively. The greater sensitivity of the dormant than of the activated spores to urethane might be interpreted to mean that the dehydrogenase systems are limiting the dormant rate. However, we have not been able to obtain more direct evidence on this point. The results reported later on carboxylase activity seem to be sufficient to explain the respiratory increase following activation, without the necessity of a change in dehydrogenase

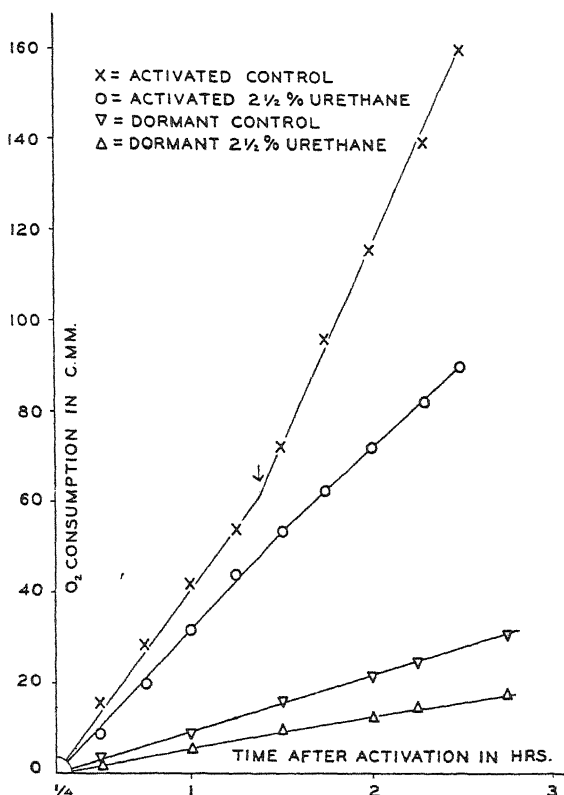


FIG. 3. The effect of 2.5 per cent. ethyl urethane on the respiration of dormant and activated spores. The arrow indicates germination; the poisoned activated spores did not germinate. 10.1 mg. of spores per vessel in the activated experiment, and 40.4 mg. of spores per vessel in the dormant experiment.

systems. The low sensitivity of activated spores to urethane remains without explanation.

Since spores may undergo activation and germination in distilled water, it is clear that the amount of stored food is not limiting the respiration of dormant spores. However, it is possible that though sufficient stored substrate is present, the rate at which it undergoes hydrolysis, phosphorylation, or other anaerobic reactions may be limiting the rate of respiration. If this

were the case, the addition of the proper intermediate substrate should cause a marked increase in the respiratory rate of dormant spores. It is seen from the results in table VII that the only substrates which have any

TABLE VII

EFFECTS OF SUBSTRATES ON OXYGEN CONSUMPTION OF DORMANT SPORES

VALUES GIVEN AS -  $Q_{O_2}$ 

SUBSTRATE	CONCENTRATION	SUBSTRATE RESPIRATION	CONTROL	PERCENTAGE STIMULATION
Glucose .....	M/50 .....	0.345	0.355	- 2.8
Sodium hexosediphos- phate .....	M/50 .....	0.344	0.355	- 3.2
Acetaldehyde .....	M/50 .....	0.707	0.355	99.2
Above three together .....	M/50 .....	0.737	0.355	107.6
Ethyl alcohol .....	M/20 .....	1.150	0.529	117.5
Acetaldehyde .....	M/50 .....	1.152	0.529	117.8*
Alcohol .....	M/20 } .....	1.178	0.529	122.8
plus acetaldehyde .....	M/50 }			
Methyl glyoxal .....	M/50 .....	0.420	0.357	15.0
Sodium succinate .....	M/20 .....	0.550	0.528	4.16
Pyruvic acid .....	M/20 .....	0.296	0.273	8.40
Sodium glycerophos- phate .....	M/20 .....	0.286	0.273	4.78
Sodium acetate .....	M/40 .....	0.301	0.303	- 0.6

\* Stimulations as high as 228% have been obtained at M/20, and 297% at M/50 acetaldehyde.

appreciable effect are acetaldehyde and ethyl alcohol. The increase caused by these compounds is not additive. Though the stimulation in respiration caused by these compounds is marked, it is far short of the stimulation induced by heat treatment. The possible significance of acetaldehyde and alcohol stimulation will be discussed later. It is seen from diagram II that alcoholic fermentation depends upon the presence of glucose, hexosediphosphate, and acetaldehyde. In an experiment these substances were added to one vessel containing dormant spores, and each substance independently to other spores, and the oxygen consumption followed. The increased oxygen consumption was no larger than was obtained from acetaldehyde alone, as may be seen from table VII. The failure of the added substrates to bring about respiratory stimulation may be due to impermeability of the spores or because the proper substrate or combination of substrates has not been found. The results obtained above with added substrates have not ruled out the possibility that the respiratory block is located in the anaerobic reactions which act on glycogen and which form the actual respiratory substrate.

ACTION OF SODIUM FLUORIDE, IODOACETATE, AND IODOACETAMIDE ON  
OXYGEN CONSUMPTION

BLACKMAN'S theory of plant respiration postulates that the first step in aerobic respiration is the conversion of carbohydrates to trioses or triose-phosphates in reactions essentially similar to those of alcoholic fermentation. This theory has recently been reviewed by TURNER (32). One approach to the possible location of the respiratory block between glycogen and the actual respiratory substrate is to study the effect of certain poisons, known to inhibit alcoholic fermentation and muscle glycolysis, on oxygen consumption. In this section we report some results on the action of sodium fluoride, iodoacetate, and iodoacetamide on oxygen consumption. We cannot say definitely what steps of the respiratory mechanism are inhibited by these poisons, but it is possible that the same reactions which are poisoned in anaerobic metabolism are also inhibited in respiration, even though the concentrations required to inhibit respiration are higher than those which inhibit fermentation. The question has been discussed at some length with special attention to iodoacetate by TURNER (32).

Sodium fluoride poisons alcoholic fermentation and has been shown by LOHMANN AND MEYERHOF (15) to poison the breakdown of phosphoglyceric acid to pyruvic acid and  $H_3PO_4$  (see diagram II). It is not claimed that this is the only reaction poisoned by fluoride. LUNDSGAARD (16) has shown that iodoacetate poisons muscle glycolysis and alcoholic fermentation, and it has been shown by MEYERHOF AND KIESSLING (20, 21) that it does not inhibit the breakdown of phosphoglyceric acid to acetaldehyde and  $CO_2$ , but that it does inhibit reactions 2 and 7 of diagram II. It is possible that other reactions may also be poisoned by iodoacetate. It is well known that iodoacetate combines with  $-SH$  groups of glutathione (4, 23) and proteins (8, 24), but the work of SMYTHE (27) indicates that its inhibition of fermentation is not due to destruction of  $-SH$  groups. GODDARD (7) has shown that iodoacetamide inhibits respiration of *Neurospora* spores, SMYTHE (27) that it inhibits alcoholic fermentation, and STANNARD (28) that it inhibits muscle glycolysis. The mechanism of the action of this compound is unknown, though it is established that it reacts rapidly with  $-SH$  groups (8, 27). The results obtained with these three poisons on the spores are shown in table VIII. The results of using iodoacetate and iodoacetamide on activated and germinating spores are recalculated from an earlier paper (7).

The great sensitivity of all phases of the respiration to sodium fluoride is the most interesting result from these experiments. If the respiration is over pyruvic acid, this result is to be expected, but this does not explain the inhibition of the respiration of ethyl alcohol and acetaldehyde by fluoride. It is possible that neither of these compounds is directly undergoing oxidation, but they may be entering into dismutation reactions similar to

TABLE VIII  
EFFECTS OF POISONS AND SUBSTRATES ON OXYGEN CONSUMPTION OF SPORES  
VALUES AS  $-Q_{O_2}$

POISON	SUBSTRATE	DORMANT			ACTIVATED			GERMINATING		
		CONTROL	EXPERIMENTAL	PERCENT-AGE INHIBITION	CONTROL	EXPERIMENTAL	PERCENT-AGE INHIBITION	CONTROL	EXPERIMENTAL	PERCENT-AGE INHIBITION
NaF M/10	None	0.32	0.067	81.5	5.08	1.61	64.7	15.75	0.76	95.2
NaF M/10	Acetal. M/50	0.34	0.19	73.8†	—	—	—	—	—	—
None	“	0.34	0.70	—	7.31	7.71	—	14.4	16.7	—
NaF M/10	Alcohol M/20	0.54	0.15	78.6†	—	—	—	—	—	—
None	“	0.54	0.89	—	—	—	—	—	—	—
Iodoacetate M/200	—	0.47	0.46	2.0	4.07	4.11	-1.0	7.36	3.46	53
Iodoacetate M/100	—	0.47	0.43	8.5	—	—	—	—	—	—
Iodoacetamide M/200	—	0.50	0.30	40.0	4.07	3.70	8.2	7.36	2.43	67

\* In no case did the poisoned spores germinate. These figures are obtained by comparing germinating controls with poisoned cells, at the same time periods after activation.

† Inhibition calculated against the rate with acetaldehyde from the experiment below.

‡ Inhibition calculated against the rate with alcohol from the experiment below.

reactions 6 and 7 of diagram II. This would seem probable enough for acetaldehyde but much less probable for alcohol, unless it is first converted to acetaldehyde.

The respiration of the germinating spores is sensitive to iodoacetate (pH 5.4) and the spores are rapidly killed by the poison. The oxygen consumption of dormant and activated spores is unaffected by iodoacetate and it is probable that it does not penetrate the cells. If dormant or activated spores stand for an hour in iodoacetate (0.005 M) and are then washed on the centrifuge, they germinate normally. The respiration of dormant and germinating spores is poisoned by iodoacetamide, while the activated spores are unaffected (see table IX). This cannot be due to failure to penetrate the

TABLE IX

ANAEROBIC CO<sub>2</sub> PRODUCTION OF ACTIVATED SPORES IN THE PRESENCE OF SODIUM FLUORIDE, IODOACETAMIDE, AND ETHYL URETHANE

MEDIUM	$Q_{CO_2}^{N_2}$	PERCENTAGE INHIBITION
Control .....	5.30	
NaF M/10 .....	1.44	73.0
Control .....	7.18	
Iodoacetamide M/200 .....	5.82	19.0
Iodoacetamide M/1000 .....	7.07	1.5
Control .....	8.13	
Ethyl urethane 2½ per cent. ....	7.35	10.0

\*  $Q_{CO_2}^{N_2} = \frac{\text{cu. mm. CO}_2 \text{ per hour}}{\text{dry wt. mg.}}$  (under anaerobic conditions).

$Q_{CO_2}^{N_2}$  and percentage inhibition calculated on the linear part of the curve.

activated spores, for if activated spores are exposed to iodoacetamide and then washed in the centrifuge, the respiration proceeds normally until the time when the controls germinate; then the respiration of the poisoned washed cells falls off and no germ tubes are formed. There seems to be a real difference in the respiratory mechanisms of dormant and germinating spores on the one hand and of activated spores on the other, with reference to iodoacetamide poisoning. No explanation of this effect seems possible at this time.

#### ANAEROBIC CO<sub>2</sub> AND CARBOXYLASE ACTIVATION

The marked inhibition of the oxygen consumption by sodium fluoride suggested that reactions similar to those occurring in alcoholic fermentation might be part of the respiratory scheme. The best approach to such reactions seemed to be through a study of the anaerobic CO<sub>2</sub> production. Re-

sults typical of many experiments are shown in fig. 4. It may be seen from the figure that no measurable anaerobic  $\text{CO}_2$  is liberated by the dormant spores. Even when 40 mg. of dry weight of dormant spores were used, no  $\text{CO}_2$  evolution was detected which was greater than the experimental error ( $\pm 1.0$  cu.mm. per hour). The activated spores evolved  $\text{CO}_2$  anaerobically quite rapidly as may be seen in fig. 4 and table IX. The rate of anaerobic  $\text{CO}_2$  production by the activated spores is linear for a short period after activation and gradually falls to zero at about three hours after the heat

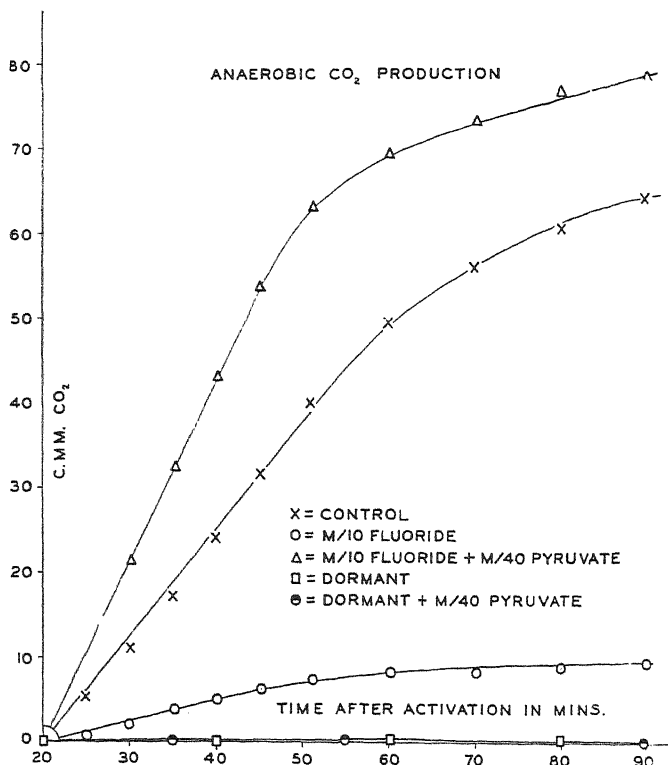


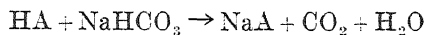
Fig. 4. Anaerobic  $\text{CO}_2$  production of dormant and activated spores. The effect of 0.1 M NaF on inhibition of  $\text{CO}_2$  production of activated spores. The effect of pyruvate and NaF together on activated spores and the failure of pyruvate to affect dormant spores. 19.5 mg. of spores per vessel.

treatment. The rate of anaerobic  $\text{CO}_2$  formation by activated spores during the linear phase is about the same as the rate of oxygen consumption. Compare tables I and IX. Though the dormant spores liberate no  $\text{CO}_2$  under anaerobic conditions, it was expected that they would produce  $\text{CO}_2$  aerobically. Aerobic  $\text{CO}_2$  production was measured by WARBURG's indirect method (see DIXON, 5) with the suspension in equilibrium with an atmos-



phere of 5 per cent.  $\text{CO}_2$  and 95 per cent. oxygen. Only a few experiments were made, and a typical one gave a  $Q_{\text{CO}_2}^{\text{O}_2}$  of 0.59 and a respiratory quotient of 0.85  $\left( Q_{\text{CO}_2}^{\text{O}_2} = \frac{\text{cu.mm. CO}_2 \text{ evolved per hr., in the presence of O}_2}{\text{mg. dry weight}} \right)$ .

Formation of anaerobic  $\text{CO}_2$  is often accompanied by formation of alcohol or organic acids. No attempt has been made to determine whether the activated spores produce alcohol under anaerobic conditions. Acid formation may be determined manometrically by the displacement of  $\text{CO}_2$  from a bicarbonate buffer according to the following equation:



Under anaerobic conditions the difference in  $\text{CO}_2$  liberated from bicarbonate buffer and from phosphate buffer is a measure of the acid formed. We carried out such an experiment with activated spores in M/60 phosphate buffer with pH 5.4 and in 0.0025 M  $\text{NaHCO}_3$  in equilibrium with 5 per cent.  $\text{CO}_2$ , with pH 7.4. The average from two vessels was 52.8 c.mm. in one hour in phosphate buffer, and 52.5 cu.mm. in bicarbonate buffer. We may be sure that under anaerobic conditions the activated spores do not form any acid.

If the block in anaerobic  $\text{CO}_2$  production of the dormant spores was anterior to pyruvic acid formation (see diagram II), it was to be expected that the addition of pyruvic acid to dormant spores would induce anaerobic  $\text{CO}_2$  production. The pyruvic acid solution used was half neutralized, so that the  $\text{pH} = \text{P}_{\text{ka}} = 2.5$ , and one half of the acid was present in undissociated form. Under these conditions (and in unneutralized pyruvic acid or in sodium pyruvate solutions), no anaerobic  $\text{CO}_2$  could be detected from dormant spores (see fig. 4). This failure of dormant spores to produce  $\text{CO}_2$  from pyruvate suggested that there was no active carboxylase in dormant spores.

Experiments on the effect of three poisons on anaerobic  $\text{CO}_2$  production are shown in table IX. In each case the  $Q_{\text{CO}_2}^{\text{N}_2}$  and the percentage inhibition were calculated on the linear part of the curve (fig. 4). The results with NaF are striking, and are in agreement with the MEYERHOF-KIESSLING theory of alcoholic fermentation. This experiment and the one which follows make it clear that the anaerobic  $\text{CO}_2$  is not merely the physical release of bound  $\text{CO}_2$  due to heating, nor to the escape of previously formed  $\text{CO}_2$  due to changes in cell permeability. From the previous discussion, it seems quite clear that iodoacetamide penetrates the spores, and it was surprising that it had such a small effect on anaerobic  $\text{CO}_2$  production. The small effect of ethyl urethane is not surprising. This experiment was included

for comparison of its effect on oxygen consumption and anaerobic  $\text{CO}_2$  production.

The fact that dormant spores liberate no anaerobic  $\text{CO}_2$  even when pyruvic acid is added, and the ready production of  $\text{CO}_2$  by activated cells suggests that carboxylase is absent or inactive in dormant spores, and that this enzyme is activated on heat treatment. If this is true, we should be able to demonstrate carboxylase activity by restoring the ability of NaF-poisoned activated spores to produce anaerobic  $\text{CO}_2$  by the addition of pyruvic acid. NaF is known to poison anaerobic  $\text{CO}_2$  formation from glucose (see diagram II), but not from pyruvic acid, since it does not poison carboxylase. The increased  $\text{CO}_2$  production on the addition of pyruvic acid over that of NaF-poisoned cells should be an approximate measure of carboxylase activity. The results from one such experiment are shown in figure 4. It is clear that pyruvic acid raises the amount of  $\text{CO}_2$  production above that of the unpoisoned controls; therefore, in the controls the rate of  $\text{CO}_2$  production is not limited by carboxylase concentration, but by the rate of pyruvic acid formation. We interpret the failure of pyruvic acid to induce anaerobic  $\text{CO}_2$  production in dormant spores and its pronounced effect on NaF-poisoned activated spores as definite evidence that the enzyme carboxylase is activated (formed?) on heat treatment of the spores.

It will be recalled that heat activation as it affects germination and increased respiration is reversible; that is, under anaerobic conditions activated spores return to secondary dormancy. These de-activated spores respond to a second heat treatment in the usual manner. The falling off in the rate of anaerobic  $\text{CO}_2$  production, with or without added pyruvic acid, is surprisingly similar to the de-activation of the germination mechanism under the same conditions. An experiment was undertaken to determine whether carboxylase activation was likewise reversible. After the activation of the spores, a sample was set aside in a hanging drop (in air) for a germination test. The spores were placed in four vessels, and anaerobic  $\text{CO}_2$  production measured. After the rate had fallen off (170 min.), two vessels were removed from the bath and spore samples were taken for germination tests. (The samples were too small to affect the subsequent readings.) The two vessels were placed for twenty minutes in a thermostat bath at  $55^\circ \text{C}$ . for re-activation. After the re-activation, samples were again removed for germination tests, the vessels replaced in the bath, anaerobic conditions established, and the  $\text{CO}_2$  production again measured. During this period, readings were continued on the other two vessels. The experiment was continued for six hours after the first activation, and by this time the rate of  $\text{CO}_2$  production had fallen approximately to zero in all vessels. The experimental results are shown graphically in figure 5.

Examination of figure 5 shows that the activation, de-activation, and

re-activation of the spores as concerns anaerobic  $\text{CO}_2$  production (and presumably carboxylase activity) is closely parallel to the reversible activation-de-activation of the germination mechanism.

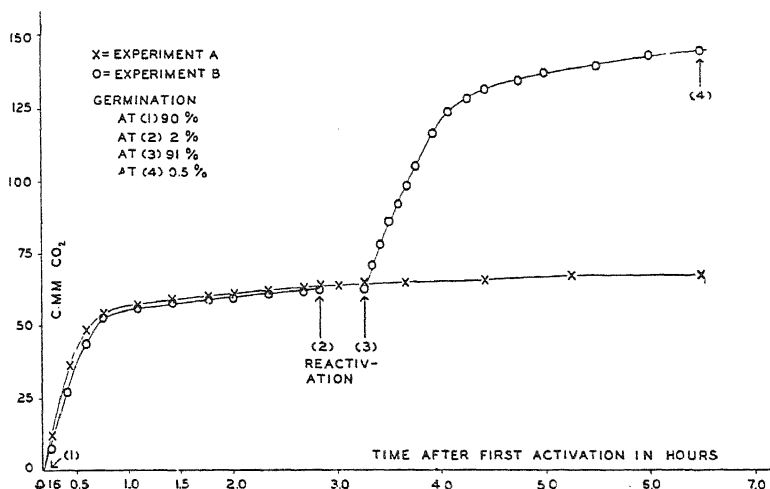


FIG. 5. The effect of a second heat treatment on the anaerobic  $\text{CO}_2$  production. In experiment A, the readings were uninterrupted. In experiment B, the vessels were removed at arrow no. 2, re-heated for twenty minutes at  $55^\circ \text{C}$ ., allowed ten minutes for temperature equilibrium, and readings commenced at arrow no. 3. Germination tests were made by removing the spores to air and counting several hours later, at each point indicated by an arrow.

### Discussion

It is possible that the respiratory rate of dormant spores is limited by cell permeability and that activation brings about a reversible change in the cell surface. The authors do not believe that this explanation is the correct one for the following reasons:

(1) The respiratory rate of the dormant spores is independent of the partial pressure of oxygen over the range of 37 to 300 mm. of mercury. This result indicates that the rate of oxygen entrance is not limiting the respiratory rate.

(2) The rate of oxygen consumption of dormant spores is independent of the partial pressure of carbon dioxide over the range of 0 to 190 mm. of mercury. This indicates that outward diffusion of carbon dioxide is not limiting the rate of oxygen consumption.

(3) The spores are not completely impermeable to carbon dioxide, for it is formed under aerobic conditions. The complete failure of dormant spores to liberate carbon dioxide anaerobically cannot be explained on a basis of permeability to carbon dioxide, for the anaerobic  $\text{CO}_2$  production would be as high as the aerobic  $\text{CO}_2$  production.

(4) The production of anaerobic  $\text{CO}_2$  by activated spores might be interpreted as the release of preformed  $\text{CO}_2$  due to a change in cell surface, but this seems improbable, for the anaerobic  $\text{CO}_2$  production is poisoned by NaF and the NaF poisoning is overcome by pyruvic acid.

(5) Ethyl alcohol and acetaldehyde cause a marked increase in the respiratory rate of dormant spores. This does not seem to be consistent with the view that the respiratory rate is limited by the cell surface.

The much greater sensitivity of the activated spores to carbon monoxide and to cyanide suggests that the activity of phaeohemin is not limiting the rate of respiration. The evidence in this paper does not definitely establish the fact that the oxidase is phaeohemin (indophenol oxidase). To prove this point, light reversal of CO poisoning would be necessary, and this is difficult to obtain with spores with black walls. Proof of phaeohemin might be obtained by the catalysis of paraphenylenediamine, but the spores appear to be impermeable to this dye. This dye could be tried on ground cells, but the authors decided to limit this paper to results obtained with intact living cells. The limited permeability of the spores has made it difficult to obtain any definite information concerning the dehydrogenase systems. It is possible that information may be gained on this point from ground cells, but how useful such information would be in attempting to interpret the changes which occur on activation is open to question. No information has been obtained on cytochrome, for the black spore wall has prevented spectroscopic determinations, and the impermeability of the spores has prevented the use of artificial carriers, such as paraphenylenediamine. It is possible that a portion of the dormant respiration is catalyzed by WARBURG's flavine enzyme. However, the activated respiration is probably not catalyzed by this enzyme, since the respiration catalyzed by the flavine enzyme (30) is cyanide- and carbon monoxide-stable.

Since increased respiration and normal germination are obtained when the ascospores are activated in distilled water, the respiratory block of the dormant spores cannot be caused by absence of stored food. However, it is possible that the reactions converting the stored food into the substrate actually respired may be limiting the respiratory rate. The only added substrates, out of a long list tried, which produced an appreciable increase in the rate of oxygen consumption of dormant spores were acetaldehyde and ethyl alcohol. These substrates stimulated the rate from 100 to 300 per cent., while the difference between dormant and activated respiratory rates is of the order of 1000 to 4000 per cent. The significance of the increase brought about by acetaldehyde or alcohol is not clear. The acetaldehyde and ethyl alcohol respiration is NaF sensitive. This is rather surprising, because NaF does not poison most dehydrogenase systems, nor the phaeohemin-cytochrome system. However, an old result of MEYERHOFF's (18) indi-

icates that succinic dehydrogenase is poisoned by NaF. Acetaldehyde may be oxidized by the xanthine oxidase. Ethyl alcohol will undergo a coupled oxidation by xanthine oxidase when hydrogen peroxide and catalase are present (12).

The failure of dormant spores to produce  $\text{CO}_2$  anaerobically in the presence of pyruvic acid, and the fact that pyruvic acid restores the anaerobic  $\text{CO}_2$  production to NaF-poisoned activated spores, seems clearly to establish the fact that carboxylase is inactive in dormant spores and active in heat-treated spores. The reversible activation of the system producing anaerobic  $\text{CO}_2$  on heat treatment parallels almost completely the effect of this heat treatment on germination and respiration. The absolute rate of anaerobic  $\text{CO}_2$  production during the linear portion of the curve is of the same order as the rate of oxygen consumption. It is difficult to determine whether or not the respiratory block is the absence of active carboxylase, but the agreement between carboxylase activity and the respiration and germination of the spores seems too close to be fortuitous, particularly so when it is recalled that activation and de-activation for one component corresponds with activation and de-activation for the other two. Taken all together, it seems probable that carboxylase activation by heat treatment of the spores leads to a marked increase in the respiratory rate and to germination. It is, of course, possible that other enzymes are similarly activated on heat treatment, and that the inactivity of one or several of these enzymes constitutes the respiratory block.

Since there seems to be no carboxylase activity in dormant spores, but a measurable respiratory rate, it seems clear that the respiratory system of dormant spores is not a system involving carboxylase. From the discussion presented it appears that the respiratory mechanism of the activated spores includes carboxylase as one essential step. If this interpretation is correct, we are forced to the conclusion that there are two qualitatively different respiratory mechanisms, namely, the dormant mechanism of which carboxylase is not a part, and the mechanism which comes into play after the heat treatment, which so closely parallels carboxylase activity that we may assume that carboxylase is one step in the system. The phaeohemin-cytochrome system and other enzymes may be common to the two systems.

If we accept the interpretation that carboxylase is an essential part of the respiratory mechanism of activated spores, then it seems clear that in this system anaerobic processes similar to those in alcoholic fermentation are a part of the respiratory mechanism. It is difficult to understand how carboxylase functions in the respiratory mechanism. It cannot be merely the formation of acetaldehyde, for though the addition of this substance causes a respiratory stimulation, it is far short of the maximum rate. Nor can it be merely the accumulation of pyruvic acid that limits the rate, for the addi-

tion of pyruvic acid does not inhibit the respiration of activated spores. AUHAGEN (1) has shown that a co-carboxylase is essential for carboxylase activity; activation might be the formation of co-carboxylase. If co-carboxylase were identical with a co-enzyme of respiration, the parallelism between carboxylase activity and respiration would be explained, without the necessity of assuming that carboxylase was part of the respiratory mechanism. But AUHAGEN has shown that co-carboxylase is not identical with co-enzyme, nor is it found in muscle, which lacks carboxylase but which contains a respiratory co-enzyme. To the authors' knowledge, WARBURG'S respiratory co-enzyme from horse blood has not been tried with carboxylase.

The zymase complex including carboxylase is widely distributed in the tissues of higher plants, which are never known to produce alcohol in nature. KOSTYCHEV (13) and BLACKMAN (3) assume that the function of the zymase system is to convert sugars into the respiratory substrates. The BLACKMAN theory assumes that these substrates are trioses. It is possible that carboxylase functions in the respiratory scheme in higher plants, converting three carbon atom keto acids into two carbon atom compounds which are then respired.

### Summary

1. An attempt has been made to locate the respiratory block in dormant *Neurospora tetrasperma* ascospores.
2. The respiratory rates at various partial pressures of oxygen and carbon dioxide indicate that permeability of the spores to gases is not limiting.
3. Studies on the cyanide and carbon monoxide sensitivity of dormant and activated spores indicate that phaeohemin (indophenol oxidase) activity is not limiting.
4. The dormant spores produce no  $\text{CO}_2$  anaerobically, while the activated spores have a  $Q_{\text{CO}_2}^{\text{N}_2}$  of 5 to 8, though the rate falls to zero after 3 to 4 hours.
5. It is suggested that no active carboxylase is present in dormant spores, but that carboxylase is reversibly activated on heat treatment. The activation-de-activation of carboxylase parallels the effect of activation and de-activation on respiration and germination.
6. The results in this paper are interpreted to mean that two qualitatively different respiratory systems are present, namely, the dormant system, which functions in the absence of carboxylase, and a second system, active in heat treated spores, which passes over the enzyme carboxylase. The respiratory block is then the inactivity of the enzyme carboxylase.

7. These experiments indicate that reactions similar to those of alcoholic fermentation are a part of the respiratory mechanism in *Neurospora*.

DEPARTMENT OF BOTANY

UNIVERSITY OF ROCHESTER

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# AMMONIUM NUTRITION AND METABOLISM OF ETIOLATED SEEDLINGS

LELAND BURKHART  
(WITH ELEVEN FIGURES)

## Introduction

The nitrogen metabolism of germinating seeds and young seedlings concerns itself chiefly with the decomposition of already existing proteins stored in the kernels, followed by the translocation and regeneration of the simpler substances into proteins in the meristematic and actively growing regions of the developing plant body. Studies of etiolated seedlings seem suitable for the purpose of obtaining information concerning the intermediate products of protein metabolism, since protein decomposition is favored while its regeneration is hindered in the absence of light because of the depletion of available carbohydrates.

The rôle of available carbohydrates in altering the nitrogen relations of etiolated seedlings supplied with ammonium salts has been emphasized by PRIANISCHNIKOW and his co-workers (29, 30, 31, 33, 34); however, no attempt was made to follow the changes in the carbohydrate relations quantitatively. The seedlings, grown in water cultures for a period of 10 days in the dark, were classified into three groups:

I. The cereal type (barley, maize), and the pumpkin when supplied with ammonium salts, showed increases in total nitrogen and amides, but no increase in ammonia.

II. The starchy legumes (pea, vetch) increased in total nitrogen and amides only when ammonium salts were accompanied by calcium carbonate.

III. The yellow lupine, high in protein, showed serious disturbances in the synthetic reactions, manifested by accumulation of ammonia and decrease of asparagine. Addition of calcium carbonate failed to restore the normal course of nitrogen metabolism.

PRIANISCHNIKOW deserves considerable credit for his choice of seeds with such a wide range of food reserves, which seems to have been a determinative factor in causing the different responses of the various seedlings to ammonium nutrition as reported by him. The seeds used differ greatly with respect to the relative amounts of nitrogen-free and nitrogen-containing reserves. The respective ratios used as an index to these differences are as follows: cereals, (6:1); starchy legumes, (2:1); and yellow lupine, (0.6:1).

In order to obtain additional information as to the rôle of carbohydrate reserves as affecting the response of seedlings to ammonium salts, so-called "artificial types" were employed in modifications of experiments previously mentioned. SMIRNOW (54) compared the nitrogen metabolism of etiolated

seedlings of high carbohydrate reserve (barley) and low carbohydrate reserve (yellow lupine), which were grown in sterile culture solutions. In the early stages of growth, ammonia was readily absorbed by the barley seedlings, followed by an increase in asparagine. In the later stages, when the carbohydrate reserves were exhausted (21 days), ammonia accumulated in the plants and asparagine decreased (an artificial lupine type physiologically). The presence of calcium salts promoted asparagine formation, but the processes ended sooner, as carbohydrates were more readily exhausted. The absorption of ammonium by lupine seedlings was dependent upon the presence of sugar in the medium. The addition of glucose along with the ammonium salts resulted in an "artificial physiological cereal type."

Although the results of these investigations have often been referred to in the literature, little attempt has been made to confirm these findings. The writer has previously criticized the methods employed by these workers (2). In the light of modern principles of mineral nutrition, the use of unbalanced nutrient solutions and distilled water as a control in growing their plants seems very undesirable. Calcium must play a more prominent rôle in altering the response of seedlings to ammonium salts than was realized by PRIANISCHNIKOW when he originally planned his experiments (32, 33, 38, 60). Not having separated the principal storage organs from the remainder of the seedlings, no basis was provided upon which to determine the extent of protein regeneration, all of which should be of interpretative value. The ammonia determinations are of questionable value, since the seedlings were dried prior to analysis.

With the adaptation of improved methods, this investigation involves a further study of the effects of ammonium nutrition on the carbohydrate and nitrogen relations of etiolated seedlings as altered by the type and amount of food reserves in the seed. An attempt is made to throw additional light upon certain fundamental questions: What determines the rate and amount of ammonium absorption by seedlings? How and to what extent is the ammonium utilized? What conditions are associated with so-called "ammonium injury"?

### Materials and cultural methods

#### CHOICE AND COMPOSITION OF SEEDS

In this study it seemed desirable to employ seeds of species representing a wide range in ratios of non-nitrogenous reserves to nitrogenous reserves. Seeds were chosen in which the cotyledons served as the storage organs, thus minimizing morphological variations. Seeds of one species, with the desired range of food reserves in the various varieties, would constitute more nearly an ideal choice; however, such are not available. Variations inherent in the species of different genera might be somewhat minimized by selecting seeds

from one family, or better, one genus. The seeds used in this investigation are of the legume family except the pumpkin which was included for further study as it was found in an earlier study to respond favorably to ammonium nutrition (2). The peanut was employed with the possibility that it might compare favorably with the pumpkin on the basis of the similarity in the chemical composition of the seeds (table I), thereby having the desired range of food reserves within one family.

The following seeds were chosen:

- Pumpkin: *Cucurbita pepo* (Small Sugar variety), representing the cereal type; high oil-low protein reserves.
- Peanut: *Arachis hypogea* (Spanish variety), possible representative of the cereal type in the legume family; high oil-low protein reserves.
- White lupine: *Lupinus albus*, representing a possible intermediate between the above types and the yellow lupine; high hemicellulose—intermediate in protein reserves.
- Yellow lupine: *Lupinus luteus*: PRIANISCHNIKOW's third type; low hemicellulose-high protein reserves.

The compositions of the kernels (seed coats removed from seeds) as determined during this investigation are presented in table I. Ether extract was

TABLE I  
ANALYSIS OF SEEDS

DETERMINATIONS	PUMPKIN	PEANUT	WHITE LUPINE	YELLOW LUPINE
Weight of 100 seeds (gm.) .....	16.65	52.08	52.47	11.52
Weight of 100 kernels (gm.) .....	13.16	50.90	43.80	8.72
Moisture in kernels (per cent.) .....	4.20	4.90	6.18	6.14
Dry weight of 100 kernels (gm.) .....	12.61	48.41	41.10	8.18
Percentage composition of kernels in terms of dry weight				
	%	%	%	%
Insoluble nitrogen .....	5.39	5.16	6.80	9.26
Soluble nitrogen .....	0.22	0.24	0.53	0.29
Ether extract .....	50.27	46.71	13.10	9.05
Starch .....	—	6.69	—	—
Insoluble acid hydrolyzable polysaccharides .....	2.88	2.84	17.04	7.76
Reducing sugars .....	0.13	0.08	0.08	0.12
Sucrose .....	3.64	2.32	3.76	1.50

determined (1) on the kernels after grinding and drying at 80° C. The methods employed in the analyses are described in a following section.

## GERMINATION

The seeds were carefully selected for uniformity in size, and all those showing defects were discarded. At least 5 times as many seeds as were to be used in the experiments were germinated after preliminary treatment with 0.2 per cent. Uspulun for 20 minutes, and soaking for 2 hours in one-fifth strength nitrogen-free nutrient solution in the case of the pumpkin and white lupines. The peanuts were found to germinate better without Uspulun treatment. The yellow lupines were treated for 1 hour with concentrated sulphuric acid with subsequent washings in lime water. The germination was increased by this treatment from 15 to 90 per cent. with much more uniformity in rate of germination. After soaking for 2 hours, the seeds were placed in glass moist chambers on cellucotton kept nearly saturated with one-fifth strength nitrogen-free nutrient solution. The germination was improved by renewal of the air in the germinators at 12-hour intervals. By means of the above precautions, vigorous young seedlings free of bacteria and molds were obtained in three to four days, depending upon the species. Germination was carried out in the dark room (free of gas and laboratory fumes) in which the subsequent experiments were carried out ( $25^{\circ}$ – $26^{\circ}$  C.). The young seedlings at the above mentioned stage were selected for uniformity (radicles 3 cm. in length) and transferred to the culture solutions. Analysis of the seedlings at this stage are presented in table II. The cotyledons were analyzed apart from the remainder of the seedlings. No attempt was made to analyze the plumules separately since these made up but a small fraction. Hence the term "roots" includes the plumules with the comparatively large and fleshy radicles. From 100 to 300 seedlings were analyzed in duplicate at this stage.

## NUTRIENT SOLUTIONS

LIVINGSTON (15) has stressed the need of much preliminary investigation to determine satisfactory salt combinations, concentrations, and pH before definite progress can be made in nutritional studies. In such studies complications arise; for an "optimum solution" varies among different species for a given stage of growth. Furthermore, environmental conditions alter the optimum solution for a given species and stage of growth. For example, an optimum solution for a normal seedling may be expected to differ materially from that for an etiolated seedling. It is evident that the choice of a suitable nutrient solution requires many considerations.

Just as FIRSCHLE (26, 27) has recognized the importance of the interaction of various factors on plant response to pH, one thinks of the interionic and other interacting factors as certainly affecting the response of etiolated seedlings to ammonium salts. HOAGLAND (8), LUNDEGÅRDH (16), and STEWARD (56) have briefly reviewed some of the recent papers pertaining to the various relationships involved in mineral nutrition.

TABLE II  
ANALYSIS OF GERMINATED SEEDLINGS AT THE BEGINNING OF EXPERIMENTS WHEN TRANSFERRED TO NUTRIENT SOLUTIONS  
(WEIGHT PER HUNDRED SEEDLINGS)

DETERMINATIONS	PUMPKIN		PEANUT		WHITE LUPINE		YELLOW LUPINE	
	COTY-LEDONS	ROOTS	COTY-LEDONS	ROOTS	COTY-LEDONS	ROOTS	COTY-LEDONS	ROOTS
Fresh weight .....	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Soluble solids .....	23.2	18.0	82.5	28.2	110.0	22.2	22.1	10.0
Insoluble solids .....	0.71	0.19	4.50	1.81	9.30	2.24	1.42	0.46
	10.11	0.40	41.66	1.73	13.75	1.41	6.02	0.13
	COTY-LEDONS	ROOTS	COTY-LEDONS	ROOTS	COTY-LEDONS	ROOTS	COTY-LEDONS	ROOTS
Insoluble nitrogen .....	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Soluble N .....	635.4	22.3	1594.0	346.4	2341.0	90.1	629.2	24.2
Amino N .....	42.9	13.6	337.5	246.0	326.0	80.0	101.5	33.8
Amide N .....	24.3	9.0	108.8	96.4	114.0	43.0	55.2	18.1
Ammonium N .....	7.2	3.4	32.0	24.0	32.0	35.0	27.0	10.4
Reducing sugars .....	1.4	0.9	5.0	3.0	9.0	3.0	1.8	0.5
Sucrose .....	19	29	60	351	73	129	5	32
Starch .....	19	6	824	480	288	129	110	15
Insoluble acid hydrolyzable polysaccharides .....	---	---	6333	961	---	---	---	---
	152	19	1150	22	5089	174	483	59

The question of pH of nutrient solutions requires serious consideration in connection with ammonium nutrition. The importance of the hydrogen-ion concentration as a determining factor in the absorption of ammonium and consequent response of plants has been stressed by PRIANISCHNIKOW, PIRSCHLE, MEVIUS and ENGEL, NAFTEL, and others (17, 18, 21, 26, 27, 35, 36, 37). In general, it has been shown that plants, under normal conditions, utilize ammonium most efficiently from a neutral or slightly alkaline medium; but the alkaline conditions are unsatisfactory because complications arise (precipitation, etc.). TIEDJENS (58) has indicated that pH values of 6.0–6.5 for ammonium nutrition with constantly flowing cultures were necessary for most satisfactory assimilation in the plant.

From preliminary trials the following salt combination on a partial volume-molecular basis proved suitable:

$(\text{NH}_4)_2\text{SO}_4$ .....	0.006 M
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ .....	0.004 M
$\text{KH}_2\text{PO}_4$ .....	0.004 M
$\text{CaCl}_2$ .....	0.004 M

A pH of 6.2 was obtained by the addition of N/10 NaOH. Proper aliquots of molar single salt stock solutions and the alkali were added to distilled water and diluted to volume just before using. The distilled water was allowed to come to dark room temperature before using. Traces of iron and boron were added. The control nutrient solution was made up in the same manner except that the ammonium sulphate was omitted.

#### CONTINUAL FLOWING CULTURES AND AERATION

As culture vessels 4-gallon glazed earthenware jars were used. Over each jar was placed a zinc galvanized wire net ( $\frac{1}{4}$ -inch mesh) well coated with paraffin which served as an efficient support for the seedlings. A modification of the STAHL and SHIVE system of continuous renewal of nutrients (55) was adapted for use with these culture vessels. Five-gallon glass bottles calibrated at 15 liters were employed as supply and reception vessels. The rate of flow of nutrient solutions was so regulated as to allow the passage of 10 liters daily through each culture vessel, thus maintaining a constant pH. The drip solution was reused once after readjusting the pH to 6.2. The cultures were continually aerated with ammonia-free air (compressed air passed through a dilute sulphuric acid trap). The rate of aeration was such that the solution was kept in slow motion. This aeration treatment resulted in noticeably better root development. At the beginning of each experiment 250 young seedlings, selected for uniformity as previously described, were transferred to each of the 6 culture vessels (3 ammonium and 3 controls).

## Methods of chemical analysis

### FRACTIONATION OF PLANT ORGANS

The seedlings at each sampling were separated into stems, cotyledons, and roots. In order to make more efficient use of the plant material, the lower one-third of the hypocotyl was included with the roots in all samplings. Fifty to one hundred seedlings were sampled at each date of sampling. After obtaining fresh weights, without delay the plant material was minced into small sections (2–3 mm.) and transferred to Erlenmeyer flasks. Sufficient hot 95 per cent. alcohol was added to bring the final concentration of alcohol to 60 per cent. The contents were heated on the steam bath to gentle boiling for 20 minutes, and allowed to cool to room temperature before filtering.

### EXTRACTION

Considerable difficulty was experienced in obtaining complete extraction of the soluble nitrogen with 80 per cent. alcohol, especially in the case of the white lupines (containing large amounts of asparagine) from which, even after 10 extractions, the asparagine was not completely removed. This difficulty was overcome by reducing the alcohol concentration to 60 per cent. A lower concentration resulted in filtration difficulties, especially in cotyledon material of lupines. It has previously been pointed out by DENNY (6) that unless the plant material is distinctly acidic there is little justification for employing  $\text{CaCO}_3$  to prevent the possible hydrolysis of sucrose. In some preliminary studies it was found that young seedlings containing large amounts of sucrose, when extracted with and without the aid of  $\text{CaCO}_3$ , gave comparable results. Accordingly, it seemed best not to include  $\text{CaCO}_3$ ; for its inclusion results in certain complications of analysis and may possibly render extracts sufficiently alkaline to result in a loss of ammonia (if present in appreciable quantities) during the removal of alcohol to obtain water extracts for nitrogen fractions. Eight to nine extractions at 6-hour intervals secured complete extraction in 2 to 3 days. After the first extraction the residue in the flasks were, in the subsequent extractions, covered with hot 60 per cent. alcohol, heated to gentle boiling for 5 minutes and allowed to cool before filtering. After the extractions were completed and the extracts diluted to a convenient volume, nitrogen fractions were determined without delay. Thus any changes which seem to occur in nitrogen fractions during storage of alcoholic extracts (57, 63, 64) were reduced to a minimum.

### ANALYSIS OF EXTRACTS

Solids and soluble nitrogen were determined on suitable aliquots.

SOLUBLE NITROGEN FRACTIONS.—The alcohol was removed from suitable aliquots, the water suspension cleared, and made to volume (2).

Ammonium nitrogen was determined on 20-cc. aliquots of the water

TABLE III

CONCENTRATION OF AMMONIUM NITROGEN IN STEMS AND ROOTS OF AMMONIUM-NOURISHED SEEDLINGS ON FRESH WEIGHT BASIS

PERIOD OF GROWTH	PUMPKIN		PEANUT		WHITE LUPINE		YELLOW LUPINE	
	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS
3 days .....	%	%	%	%	%	%	%	%
6 " .....	0.012	0.022	0.017	0.023	0.010	0.009	0.007	0.009
9 " .....	0.025	0.027	—	—	—	—	0.027	0.020
12 " .....	—	—	0.019	0.025	0.021	0.036	—	—
14 " .....	0.044	0.045	—	—	—	—	0.045	0.045
16 " .....	—	—	—	—	0.046	0.087	—	—
18 " .....	—	—	0.027	0.030	—	—	—	—
30 " .....	—	—	0.037	0.032	—	—	—	—

extract in the Van Slyke-Cullen aeration apparatus. The aliquot was made 0.6 per cent. sodium hydroxide by the addition of 10 per cent. sodium hydroxide and aerated into 25 cc. of N/50 standard sulphuric acid, employing capryl alcohol to prevent foaming.

The ammonium-free aliquot was neutralized with glacial acetic acid added dropwise and an additional drop added to slightly acidify the mixture, which was then carefully transferred to a 50-cc. volumetric flask, the tube being carefully rinsed several times into the flask with small portions of distilled water. The flask was made to volume and the amino nitrogen determined on 2.5-cc. aliquots in the Van Slyke amino-nitrogen apparatus.

Ten-cc. aliquots of the cleared water extract were hydrolyzed (25), cooled, nearly neutralized with 1.5 cc. of 50 per cent. NaOH and allowed to recool.

TABLE IV

CONCENTRATION OF TOTAL SUGARS IN STEMS AND ROOTS OF AMMONIUM-NOURISHED SEEDLINGS ON FRESH WEIGHT BASIS

PERIOD OF GROWTH	PUMPKIN		PEANUT		WHITE LUPINE		YELLOW LUPINE	
	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS	STEMS	ROOTS
3 days .....	%	%	%	%	%	%	%	%
6 " .....	0.276	0.114	1.383	0.639	0.625	0.510	0.214	0.050
9 " .....	0.062	0.042	—	—	—	—	0.006	0.009
12 " .....	—	—	0.877	0.269	0.136	0.040	None	0.003
14 " .....	0.006	0.012	—	—	0.009	0.017	—	—
16 " .....	—	—	—	—	—	—	—	—
18 " .....	—	—	0.424	0.220	—	—	—	—
30 " .....	—	—	0.040	0.010	—	—	—	—



The solution was then made neutral to neutral red, and without delay made 0.62 per cent. NaOH and aerated into 25 cc. of N/50 standard acid. This gave ammonium equivalent to ammonium plus amide nitrogen. In the light of the recent findings of PUCHER *et al.* (28), VICKERY (61, 62), STUART (57), SCHLENKER (46), DAVIDSON and SHIVE (5), HULME (11), RICHARDSON (42), TOTTINGHAM *et al.* (59), the present methods of analysis of N-fractions are seriously in need of further critical examination.

**SOLUBLE CARBOHYDRATES.**—Direct reducing sugars and sucrose were determined on cleared and dealed aliquots of the alcoholic extracts (2). A modification of the TOMPSETT method (23) was employed in all carbohydrate determinations.

**STARCH, INSOLUBLE ACID-HYDROLYZABLE POLYSACCHARIDES, AND NITROGEN.**—Starch, insoluble acid-hydrolyzable polysaccharides, and nitrogen were determined on the residues (2). The peanut was the only species studied which contained starch. Dextrins were determined in the extracts of the peanut and included with the starch. Owing to the large sugar blank on the high grade commercial taka-diastrase distributed by the Parke, Davis Co., it was found necessary to dialyze the preparation free of sugars before use (7). In the case of the peanut cotyledons, the residues were extracted with ether before determining starch.

**EXPRESSING RESULTS.**—It seemed that the results obtained in this type of investigation would in general be expressed most significantly on the basis of weight of the respective constituents per hundred seedlings. In order more conveniently to study the various trends on a comparable basis, the data were plotted graphically, thus facilitating a picturization of the relations in figures 1 to 8. The increases or decreases in the various constituents for the particular organs of a given species, resulting from ammonium nutrition, are readily shown in these figures. Certain conditions existing in the various species are more comparable when the constituents are expressed as percentage of fresh weight (tables III and IV). In order to make a comparable study of the seedlings with respect to growth response, ammonium absorption, and ammonium utilization in relation to the food reserves of the seed, certain constituents were expressed on a comparable basis of amount of constituent per 100 gm. of dry ungerminated kernels in figures 9 to 11.

**SIGNIFICANCE OF RESULTS.**—Having carefully selected the seeds and again the young seedlings for uniformity, variations in duplicate samples of the experimental plant material owing to individual variations of the plants were reduced to a minimum (often within the limits of error of the methods of chemical analysis (1 to 2 per cent.)), by the choice of a large number of individuals (50 to 100) per sample. Differences greater than 4 to 8 per cent. between the averages of four determinations per constituent of the two series respectively were considered significant.

## Experimentation

## PLAN OF EXPERIMENTS

Preliminary experiments were performed to determine the age of the seedlings of the various species at the time when starvation symptoms were apparent as the result of ammonium nutrition. Having thus determined the starvation periods for the various species, intervening sampling periods were chosen at suitable intervals in an attempt to obtain information useful in interpreting the conditions associated with ammonium injury.

Note that when the term "ammonium injury" is used in the following discussions it is not meant to imply that ammonium as such is injurious, as some writers seem to believe, but rather to convey in a convenient manner the complex of conditions associated with plants injured by ammonium nutrition. In short, the writer prefers to consider ammonium as only *one* of the various factors bringing about such pathological conditions. To be more specific, in the cases of the ammonium injury reported in the following discussions

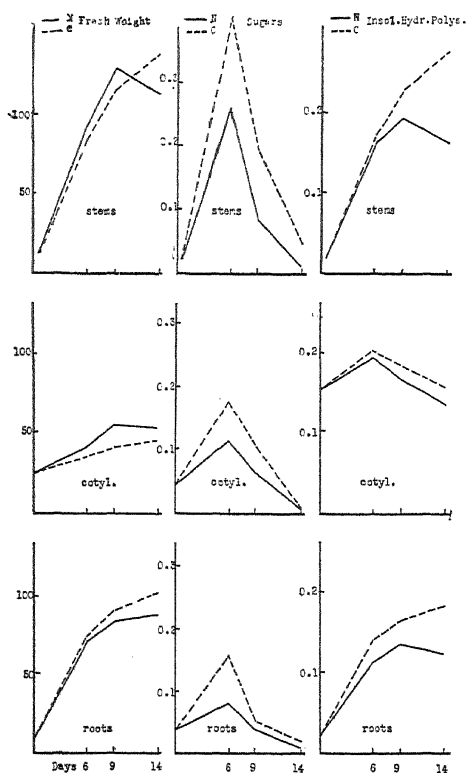


FIG. 1. Pumpkin. Fresh weights and carbohydrates in grams per hundred seedlings.

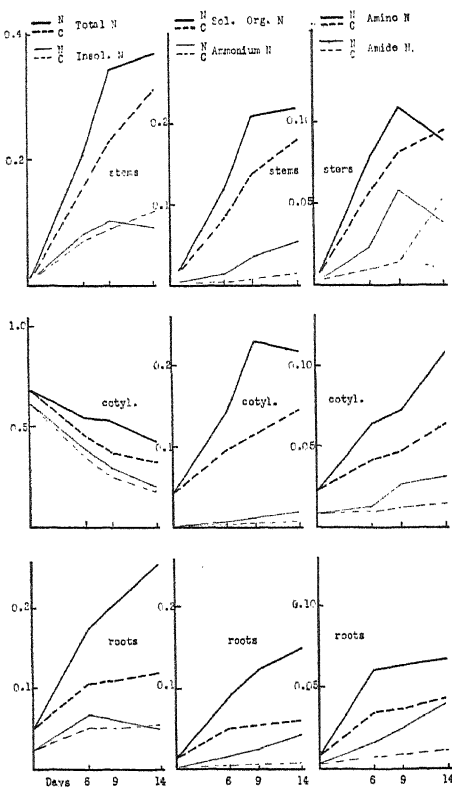


FIG. 2. Pumpkin. Nitrogen fractions in grams per hundred seedlings.

the writer prefers to consider that this condition is "injury due to extreme carbohydrate deficiency," since this would accurately express the truth; for, other conditions being favorable, including the presence of available carbohydrates, ammonium is *not toxic*.

#### EXPERIMENTS WITH PUMPKIN

The pumpkin seedlings were sampled at 6-, 9-, and 14-day periods. (The number of the days indicates the time elapsed from the date on which the young seedlings were transferred to the culture solutions.) The data obtained are graphically presented in figures 1 and 2.

A consideration of the fresh weight curves reveals the beneficial effects of ammonium on the growth of the stems and cotyledons during the earlier stages of growth. This favorable effect was previously noted (2). However, growth of roots was retarded. Similar growth relations were noted by REID (41) when etiolated seedlings were supplied with nitrates. The pumpkin differs materially from the other seedlings in that the cotyledons become very much expanded (growth) and succulent, and serve as very efficient photosynthetic organs in the light. At 14 days, the small leaves of the ammonium-fed epicotyls showed typical carbohydrate starvation symptoms (desiccation of the small young leaves).

The sugars were rapidly depleted in all organs by ammonium nutrition (fig. 1) with simultaneous decreases in amounts of hemicellulose. Sucrose, although present in appreciable amounts at the beginning of the experiments (table II), especially in the cotyledons as in the other species, disappeared at a much more rapid rate than the reducing sugars. Starch was absent from all tissues.

**NITROGEN FRACTIONS.**—The curves (fig. 2) show the remarkably rapid rate of ammonium absorption and utilization. In the early stage, protein formation especially in the roots was favored by ammonium nutrition, but not at the expense of the protein reserves of the cotyledons. It is of interest to note that the root development was retarded, even though protein formation was favored. These relations are to be expected since it has been repeatedly demonstrated by various investigators that root development is favored by higher carbohydrate conditions. In the intermediate stage (9-day) protein formation was retarded in the roots with a simultaneous increase in the stems, although not appreciable. Ammonium nutrition resulted in a sparing action on the reserve proteins at this stage. Amides strikingly had accumulated at this period while ammonium also accumulated in appreciable quantities in all organs. At the period of carbohydrate starvation (14 days) the synthetic processes were seriously disrupted as evidenced by the decrease in amino acids and amides. However, these conditions were just the reverse in the roots. The accumulation of amino nitrogen with a simultaneous

decrease in total soluble organic nitrogen in the ammonium-nourished cotyledons suggests that the amino acids accumulate at the expense of the more complex soluble compounds (polypeptides, etc.).

#### EXPERIMENTS WITH PEANUT

The seedlings were sampled at 6, 12, 18, and finally at 30 days when ammonium injury was apparent (starvation symptoms—desiccation of the older leaflets). Control seedlings were grown for 40 days when starvation symptoms were evident similar to those observed in the ammonium-nourished seedlings. There was no nodule formation in either series of the peanuts and lupines, and no evidence of nitrogen fixation was obtained. As is shown in figure 3, the seedlings did not respond significantly to ammonium nutrition during the early stages. The differences in amount and concentrations of sugars between the two series were amplified as the seedlings approached starvation especially in the roots. Somewhat similar trends were also appar-

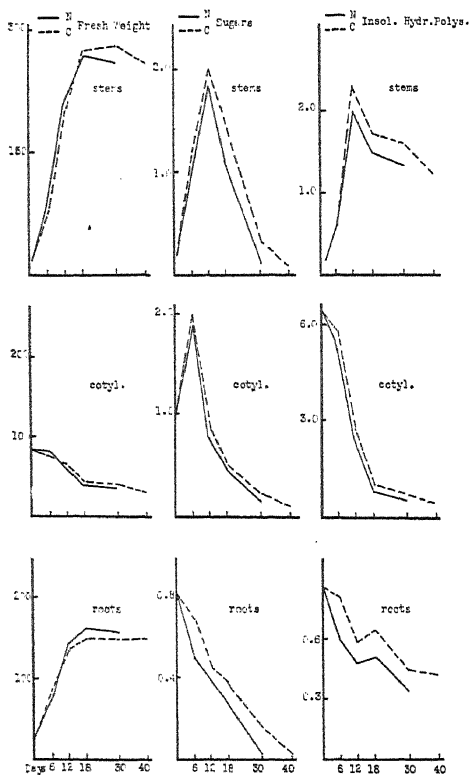


FIG. 3. Peanut. Fresh weights and carbohydrates in grams per hundred seedlings.

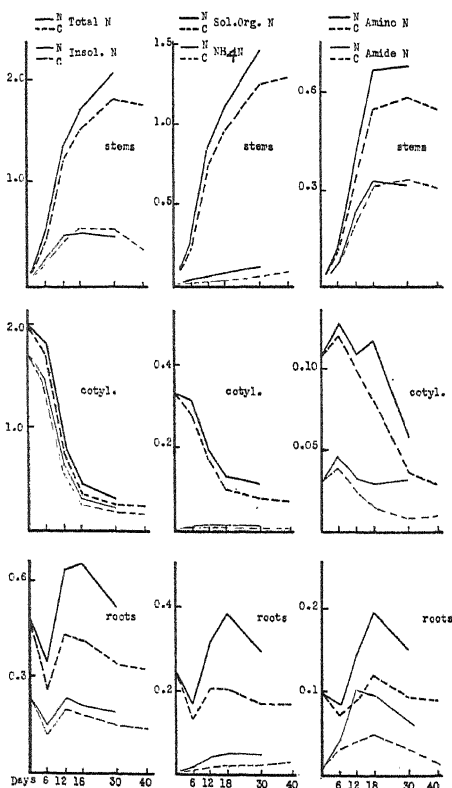


FIG. 4. Peanut. Nitrogen fractions in grams per hundred seedlings.

ent in the hemicelluloses and to a less extent in the starch in the cotyledons and roots. The trends in the nitrogen relations (fig. 4) were not as striking as those noted in the pumpkin. The initial drop in the nitrogen-fraction curves in the roots is due to the fact that the plumules and upper hypocotyls were not separated from the radicles at the initial sampling period. The relative rates of absorption and utilization of ammonium were considerably less than those noted in the pumpkin. Ammonium nutrition resulted in significant increases in protein at 12 days, but not at the expense of the protein reserves, owing to presence of adequate carbohydrate reserves. Marked increases in the soluble organic nitrogen fractions in the roots were favored by ammonium nutrition until the 18-day period. There seems to have been considerable translocation of the organic nitrogen from the roots to the tops in both series during the 18- to 30-day period. The decided increase in soluble organic nitrogen of the stems during this period seems to have been in part due to the movement of organic nitrogen from the cotyledons. The leveling of the organic nitrogen curves during this period suggests that the marked increase in the stems must have been caused by inward movement of organic nitrogen from the roots. Although the synthetic processes were materially inhibited during the carbohydrate starvation period (18 to 30 days) there seems to have been no serious breakdown of total organic nitrogen or of amides by ammonium nutrition even at the point of serious carbohydrate deficiency (fig. 4).

#### EXPERIMENTS WITH WHITE LUPINE

Seedlings were sampled at 6, 12, and finally at 16 days, when ammonium injury appeared characterized by typical starvation symptoms as previously described for the pumpkin (desiccation of the leaflets). The analytical data are presented graphically in figures 5 and 6. Ammonium nutrition resulted in better growth of roots, and inferior growth of tops, which was true throughout all stages. These relations were also noted in the yellow lupine experiments. Considerable enlargement of the lower hypocotyl resulted from ammonium nutrition. This enlargement was not caused by increased secondary thickening, but by enlargement of pith and cortical cells. Better development of lateral roots was favored by ammonium nutrition. Although significant decreases in sugars were noted in all organs of ammonium-nourished seedlings the hemicelluloses were not significantly less than in the cotyledons of the control except at starvation. The synthesis of organic nitrogen from ammonium proceeded rather smoothly during the early stages in all organs, especially the roots. However, ammonium nutrition resulted in a serious disruption of synthetic processes in the carbohydrate starvation period (12 to 16 days); amides were remarkably decreased in all organs of the plants, and ammonium accumulated in considerable quantities

in the stems and roots; however, the amino nitrogen simultaneously increased in these organs. During this period amides and amino nitrogen increased in the stems and cotyledons but decreased slightly in the roots of the controls.

#### EXPERIMENTS WITH YELLOW LUPINE

Samplings were made at 3, 6, and finally at 9 days when carbohydrate starvation symptoms were apparent in the ammonium-nourished seedlings. Data obtained are presented graphically in figures 7 and 8. Very narrow differences in fresh weight of the various organs were obtained in the two series during the early stages (3 days). At 6 days the growth of the ammonium epicotyls was noticeably stunted, which is reflected in fresh weight difference in the stems in figure 7. This difference was much more pronounced at carbohydrate starvation (9 days). The depletion of sugars by ammonium nutrition was very marked at 3 days, especially in the roots, in which regeneration of hemicelluloses was materially hindered especially

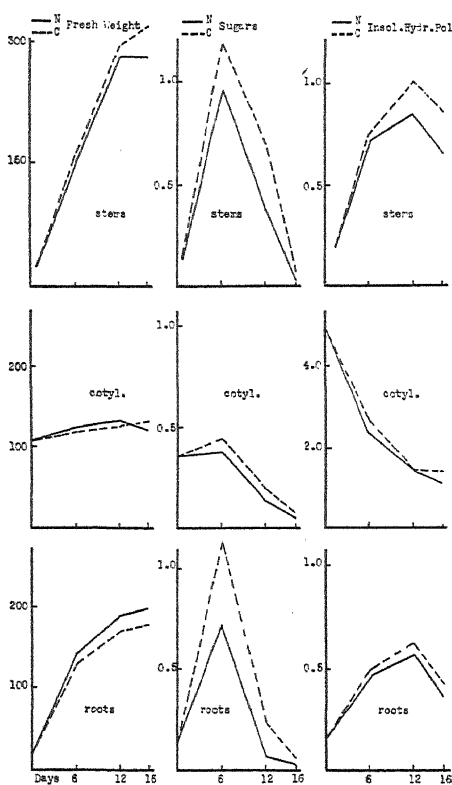


FIG. 5. White lupine. Fresh weights and carbohydrates in grams per hundred seedlings.

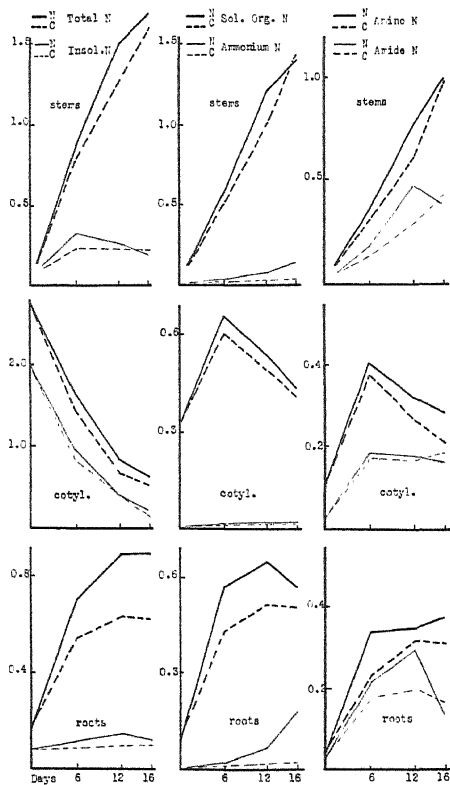


FIG. 6. White lupine. Nitrogen fractions in grams per hundred seedlings.

during later stages. Hemicelluloses seemed to have decreased in the stems during the 3- to 6-day period.

In the early stage, ammonium effected a significant increase in organic nitrogen, especially in the roots, where there was a significant increase in protein nitrogen. By the sixth day this increase in protein nitrogen had disappeared; however, during this same period there was a marked increase in amide and amino nitrogen which were favored by ammonium nutrition. This trend was not as great in the stems during this period. During the period of carbohydrate deficiency (6 to 9 days) the synthetic processes were seriously disrupted, especially in the stems, as a result for the most part of carbohydrate deficiency. Both amide and amino nitrogen decreased, and ammonium accumulated considerably. During this same period there was a remarkable increase in amide and amino nitrogen in the control stems.

These findings concerning the yellow lupine constitute a more complete story of the metabolism of the etiolated yellow lupine seedlings than the reports by PRIANISCHNIKOW and his co-workers (29, 30, 31). These investigators sampled the etiolated yellow lupine seedlings at ten days and did not make samplings at intervals during the life period. As indicated in figures 7 and 8, the life period of the etiolated yellow lupine seedlings supplied with ammonium was 9 days. After having determined the life period of the seedlings in preliminary trials, samplings were made at convenient intervals which were considered of interpretative value in respect to the various internal changes which occur during the progress of growth.

As indicated in the remarks concerning the other species of seedlings employed in these studies, a similar procedure of sampling the material for analysis was carried out. From the foregoing studies of ammonium nutrition, the following trends were noted in the various species of seedlings employed:

(1) In the early stages of growth, when available carbohydrates are abundant, ammonium is readily absorbed and utilized, resulting in protein synthesis which is especially evident in the roots. There is a tendency for growth of the seedlings to be favored by ammonium. Duration of these early stages varies considerably with the various species and seems to be associated somewhat with the type and amount of food reserves stored in the kernel.

(2) In the intermediate stages when available carbohydrates are less plentiful, although ammonium is absorbed and utilized, proteins are broken down and amides accumulate. During this period ammonium has little effect upon growth.

(3) During the carbohydrate starvation period, when available carbohydrates have become seriously depleted, ammonium is neither utilized, nor absorbed; proteins continue to be broken down; amides are decomposed; ammonium accumulates; and there may, or may not be, an increase in am-

monium nitrogen. There is a tendency for leaching of nitrogen from the seedlings to occur during this period as will be shown later. Carbohydrate starvation becomes progressively evident as characterized by cessation of growth followed by desiccation of the aerial organs. The accumulation of ammonium during this period is a clue to the breakdown of organic nitrogen compounds resulting from extreme deficiency of available carbohydrates.

#### COMPARATIVE STUDY OF SEEDLINGS

The comparative growth responses of the various species of seedlings supplied with ammonium are of interest with respect to their relationship to the food reserves. In figure 9 the fresh weights are presented on a comparable basis as a criterion of growth response.

During the early and intermediate stages of growth of the seedlings when sugars were plentiful (table IV), the ammonium absorption curve (fig. 10) closely paralleled the ammonium utilization curve (fig. 11) for the respective

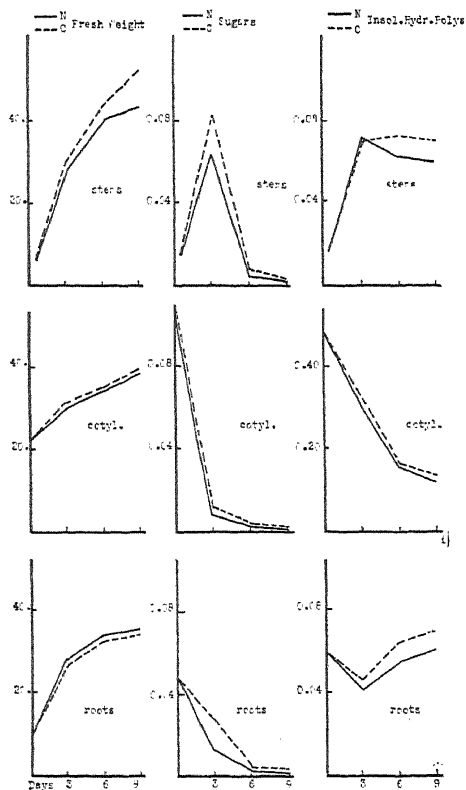


FIG. 7. Yellow lupine. Fresh weights and carbohydrates in grams per hundred seedlings.

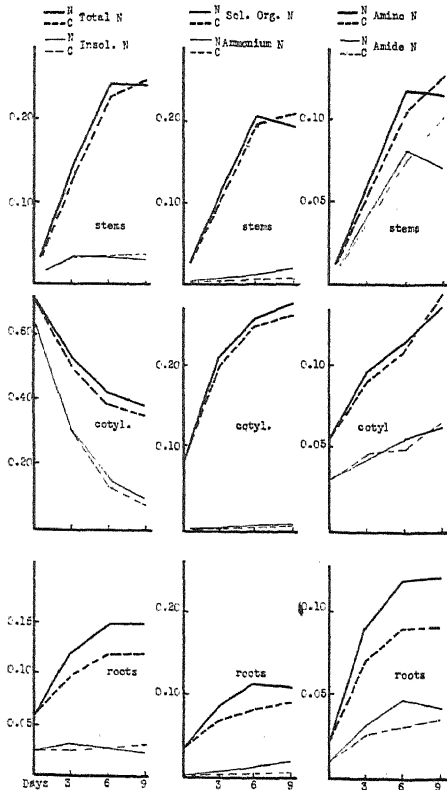


FIG. 8. Yellow lupine. Nitrogen fractions in grams per hundred seedlings.



species. During the later stages of growth as the available carbohydrate supply became exhausted (table IV), these curves diverged from each other due to the cessation of ammonium absorption in conjunction with the continued breakdown of the simple organic nitrogen compounds which results in ammonium accumulation (table III). The leaching of nitrogen from the seedlings during the carbohydrate starvation period is indicated in figure 10.

Although in the early stages of growth the concentrations of sugars in the stems and roots of the peanut seedlings are approximately six times the concentration of sugars in the stems and roots of the pumpkin seedlings (table IV), the rate of ammonium utilization by the peanut seedlings is about one-third the rate of ammonium utilization by the pumpkin seedling. This

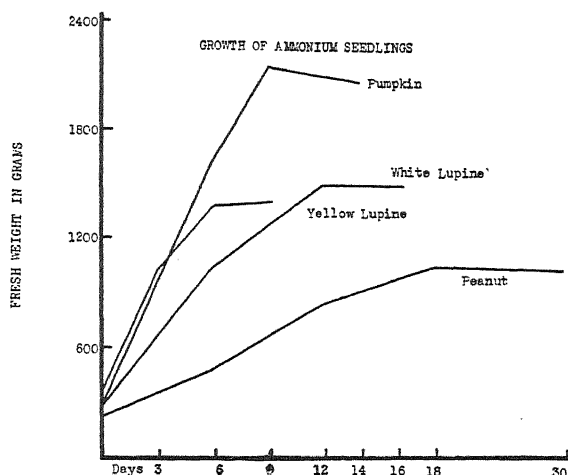


FIG. 9. Fresh weights of seedlings supplied with ammonium nitrogen (calculated on comparable basis as fresh weight in grams produced from a hundred grams of dry ungerminated kernels).

apparent inconsistency seems to be caused by inherent differences in the metabolic characteristics of the species not measured by the present chemical methods. It is difficult to understand why the peanut seedling should be so sluggish in its response to ammonium when the sugar concentration (table IV) in the actively growing seedling is so much greater than in the very responsive pumpkin seedling. The sluggishness of the peanut seedling cannot be attributed to an inferior absorption mechanism, for the concentrations of ammonium in the seedling of the peanut and pumpkin seedlings are approximately the same (table III). The behavior of the peanut seedlings, and their relatively long life period closely resemble the *Phaseolus* seedling (2) which represented the typical starchy legume type.

The apparent sluggishness of the peanut seedlings with respect to ammonium absorption and utilization seems to be associated in part with the

sluggish growth characteristics (fig. 8). On the other hand the remarkable response of the pumpkin seedlings with respect to ammonium absorption and utilization appears to be associated with the relatively rapid growth rate (fig. 8). This rapid growth rate seems to be related to the rapid conversion of the fatty reserves into sugars, which are utilized in growth and respiration but are not stored as starch, as occurs with a considerable portion of the sugars in the early stages of growth of the peanut seedlings.

The relative rates of respiration of the various species of seedlings should have thrown considerable light upon their comparative responses to am-

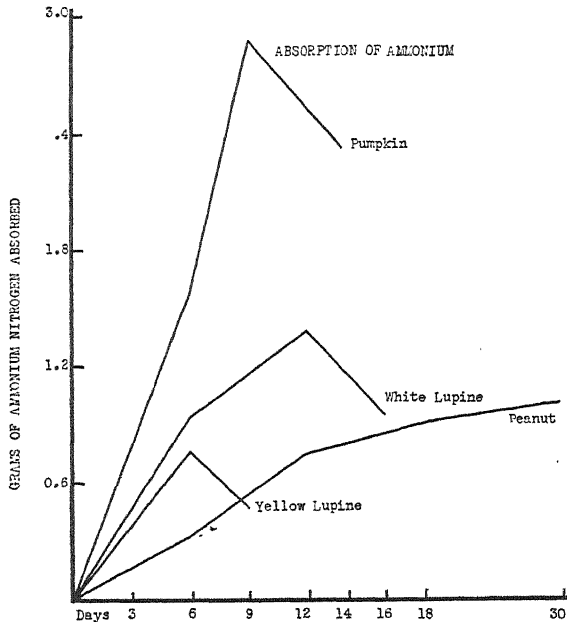


FIG. 10. Absorption of ammonium nitrogen by etiolated seedlings (calculated as difference in total nitrogen between the ammonium seedlings and control seedlings on comparable basis of a hundred grams of dry ungerminated kernels).

monium. Of special interest would be a comparative study of the respiration of the peanut and pumpkin seedlings of which the ungerminated kernels are high in oil. The rapid conversion of the oil to starch makes the peanut a starchy type, whereas there is distinctly no such conversion in the pumpkin seedlings which are so responsive to ammonium. A study of the respiration of the yellow lupine seedlings would be of interest in connection with the high protein reserve in the ungerminated kernels.

A comparative relationship of the soluble and total solids in the seedlings is presented in table V. In order more conveniently to make comparisons, the data pertaining to the solids in the seedlings have been purposely con-

densed to the form in which they appear in this table. Data pertaining to all of the species of seedlings appear for the 6-day period. And finally, data on solids at the period of extreme carbohydrate starvation, of the ammonium-nourished seedlings appear for the respective species. At the 6-day period the pumpkin seedlings increased in total solids, but markedly decreased thereafter. There seems to be no consistent trend with respect to the effect of ammonium upon the total solids. In the case of the pumpkin, however, ammonium resulted in a very significant decrease in total solids at the 14-day period. This seems to be the resultant of increased respiration because of the favorable effect of the ammonium upon the growth of these seedlings. Of

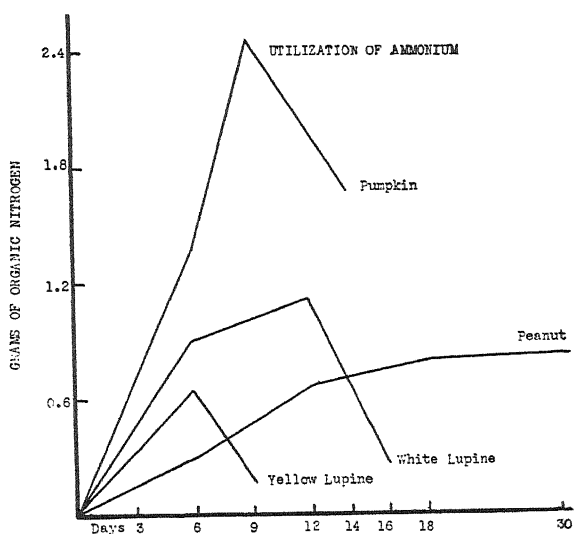


FIG. 11. Utilization of ammonium nitrogen by etiolated seedlings (calculated as difference in organic nitrogen between the ammonium seedlings and control seedlings on comparable basis of a hundred grams of dry ungerminated kernels).

the respective species, the loss of total solids during the life period was greatest in the case of the peanut, which seems to be associated with its long life period and the nature of the food reserves in its kernels.

### General discussion

A few remarks in regard to the present status of the nitrogen relationships in seedlings are now presented for the purpose of orientation. One of the most striking observations has been the accumulation of amides in shoots of growing seedlings (especially of the Leguminosae), when germinated in darkness for two or three weeks. This observation has been the subject of much discussion in recent years. In some seedlings, the proportion of amides to amino acids is fairly uniform at certain stages of germination, while at other

TABLE V

SOLIDS IN SEEDLINGS AS PERCENTAGE OF DRY UNGERMINATED KERNELS (N = AMMONIUM-NOURISHED SEEDLINGS; C = CONTROL SEEDLINGS)

PERIOD OF GROWTH	PUMPKIN		PEANUT		WHITE LUPINE		YELLOW LUPINE	
	SOLUBLE SOLIDS	TOTAL SOLIDS	SOLUBLE SOLIDS	TOTAL SOLIDS	SOLUBLE SOLIDS	TOTAL SOLIDS	SOLUBLE SOLIDS	TOTAL SOLIDS
	%	%	%	%	%	%	%	%
6 days N .....	46.7	104.6	22.6	96.4	45.9	95.9	47.8	80.8
C .....	44.7	101.6	22.8	96.6	45.4	93.0	49.4	82.8
9 days N .....	—	—	—	—	—	—	49.5	79.5
C .....	—	—	—	—	—	—	49.4	79.5
14 days N .....	36.2	81.3	—	—	—	—	—	—
C .....	32.9	87.8	—	—	—	—	—	—
16 days N .....	—	—	—	—	40.1	73.5	—	—
C .....	—	—	—	—	36.9	72.7	—	—
18 days N .....	—	—	33.6	77.8	—	—	—	—
C .....	—	—	32.2	77.9	—	—	—	—
30 days N .....	—	—	24.6	67.8	—	—	—	—
C .....	—	—	24.4	68.8	—	—	—	—

stages amides prevail. Recently PUCHER (28) and VICKERY (62) have demonstrated the widespread occurrence of glutamine in seedlings. Seedlings of legumes are rich in asparagine, while high oil-containing seeds, such as the sunflower, pumpkin, and castor bean, produce considerable amounts of glutamine. It has been shown that the amides accumulate in greater amounts than can be accounted for by direct hydrolysis of reserve proteins. Recently KLEIN and TAUBÖCK (12, 13) have emphasized the importance of basic amino acids which accumulate in considerable amounts in certain types of seeds on germination, with special attention given to arginine. Little is known concerning the interrelationships and significance of these facts.

SCHULZE (47-53) and PRIANISCHNIKOW (29-34, 39) have independently conducted a long series of investigations in which attempts were made to throw light on these phenomena. Both agree that much asparagine is formed during germination, and that under favorable conditions it is used in protein synthesis. Ammonium is formed by deaminization, probably by oxidation of amino acids. The origin of succinic acid, which they postulate plays an important rôle in asparagine formation, is somewhat obscure. SCHULZE (50) is inclined to believe that amino acids  $\rightarrow$  ammonia breakdown with subsequent formation of asparagine is to a large extent an essential process, and that asparagine is the chief source of nitrogen for protein synthesis.

PRANISCHNIKOW (29-37, 39) regards asparagine chiefly as a by-product in which form the surplus, and harmful though valuable ammonium may be stored for future use. He believes that the accumulation of amides is dependent upon a proper carbohydrate-nitrogen balance. When excess carbohydrates are present, as in photosynthesis, asparagine disappears owing to favorable conditions for protein synthesis. When this condition exists, asparagine serves as a translocatory form of ammonium and is utilized in protein synthesis. CHIBNALL is in favor of this view (3, 4). When a reasonable amount of carbohydrates is present, as in the early growth stages of etiolated seedlings, there is an accumulation of amides, for conditions are not favorable for maximum protein synthesis. But when extreme deficiency of carbohydrates is obtained, there is no opportunity for the accumulation of asparagine, owing to lack of oxidation products upon which asparagine synthesis depends. Under these latter conditions, ammonium accumulates, thereby becoming toxic. The writer seriously questions the validity of such an inference regarding the toxicity of ammonium, because it may accumulate considerably in plants without injury (14, 44). He prefers to think of the pathological condition during which ammonium accumulates as caused by an unfavorable complex of conditions in which depletion of available carbohydrates plays a predominant rôle, and that the accumulation of ammonium is probably a resulting phenomenon, but not the cause of the condition.

MURNEEK (20) in recently reviewing the rôle of asparagine and related substances in plants has not seemed to have critically evaluated the findings and conclusions of PRANISCHNIKOW. It seems that, although amides accumulate under certain conditions, there is little justification for assuming that amides serve as efficient detoxicants of ammonium. It is believed that further studies of the so-called "acid plants" (14, 43, 44) subjected to ammonium nutrition should stimulate new lines of thought in regard to these phases of nitrogen metabolism. The writer prefers to consider the accumulation of asparagine to be a resultant of certain carbohydrate-nitrogen relations and that it really does not serve a purpose in the detoxification of ammonium.

Owing to the lack of a definite understanding of nitrogen relations existing in normal seedlings, it becomes very difficult to make a satisfactory analysis of the results obtained in this investigation. Accordingly, some interpretations and generalizations in regard to the conditions brought about by ammonium nutrition in the etiolated seedlings are of necessity partially speculative. In order to grow plants under controlled conditions, they must necessarily be placed under somewhat artificial conditions. Even though the seedlings employed in this study were grown under extremely artificial conditions (40), it is believed that a proper analysis of the controlled environmental factors in conjunction with a careful consideration of the differences brought about by the variable (ammonium supply), should throw light upon

certain phases of plant metabolism which may not otherwise be obtained. From this viewpoint, this study was deemed justifiable in the light of the present confusion regarding the rôle of certain nitrogen compounds in the normal metabolism of plants.

In these experiments it was found that growth response of the various seedlings supplied with ammonium salts was in part dependent upon the nature of the food reserves. In this connection, REID (41) has mentioned some of the practical implications arising from studies of the response of etiolated seedlings to nitrogen supplied in the form of nitrates. Owing to the wide differences in the morphological characteristics, as well as to the complex hereditary tendencies inherent in the various species, it becomes somewhat far-fetched or questionable definitely to assign the different types of response (growth and certain internal conditions measurable by present chemical methods) to the type and amount of food reserves existing in the kernel. Certainly the hereditary tendencies in conjunction with modified physico-chemical relationships must play an important part in determining the responses of various seedlings to ammonium nutrition. The trends noted in these species would in no wise be expected to occur in the "acid plants" of RUHLAND and WETZEL (43, 44), especially with respect to the amide relations.

A further complication in making certain generalizations in such an investigation is the lack of knowledge as to the exact nature of the food reserves in the seeds; for example, ether extract and hemicelluloses are empirical terms assigned to groups of substances (more or less heterogeneous) about which little is specifically known. MILLER (19) gives a comprehensive historical review of the earlier investigations pertaining to the ether extract as storage material in the seeds and the changes it undergoes during germination. In this connection he (19) has studied quite extensively the changes occurring during the germination of sunflower seedlings. Little work has been done on hemicelluloses of seeds and seedlings, aside from the studies carried out by SCHULZE (1889-1910). Reserve protein is an ambiguous term, in that it is so closely interrelated to non-reserve protein that a differentiation of the two forms as they occur in the storage organs is beyond the scope of present methods of chemical analysis (22). Furthermore, little is known concerning the nature and rôle of non-protein nitrogen in the seed and seedlings, especially the "rest nitrogen."

The question as to the mechanism of ammonium absorption by plants has received but little consideration. NEVIUS (17, 18) believes that ammonium absorption increases in neutral or slightly alkaline solutions, owing to the greater degree of hydrolytic cleavage of the salts and correspondingly greater ammonia tension, which determines to a large extent the physiological effects of ammonium salts. Furthermore, he states that the injury may result from

a too rapid accumulation of ammonium under neutral or slightly alkaline conditions. NAFTEL (21) believes that ammonium alters the colloidal complex of the protoplasm which in turn affects the rate of absorption of ammonium. The study of interionic absorption, to say the least, involves many complications (PIRSCHLE, 26, 27 and NEVIUS, 17, 18). Although in accordance with absorption principles, these explanations are quite theoretical and the assumptions call for experimental verification. HOAGLAND (8), LUNDEGÅRDH (16), and STEWARD (56) have briefly reviewed some recent papers pertaining to ionic absorption. HOAGLAND and BROYER (9) believe that the absorption of ions is dependent upon metabolic activity. This view seems highly speculative. HOAGLAND (8) also emphasizes the importance of the buffering system of plant sap as a factor in ionic absorption. PRIANISCHNIKOW (36, 37) has recently shown that the rates of absorption of ammonium and nitrate by etiolated seedlings from single salt solutions of ammonium nitrate varies considerably with the pH of the culture medium and the age of the seedlings. However, no attempt was made to determine the extent of utilization. The author questions the value of such experiments in which single salt solutions are employed.

One thinks of the ammonium ion, after having entered the root of seedlings, as affecting directly or indirectly innumerable processes which in turn have a direct bearing on metabolism and growth. Various physico-chemical relationships in the cellular structure must be altered by the ammonium ion, such as permeability, translocation, pH, and interionic relations within the plant tissue. HOLLEY *et al.* (10) have shown that ammonium definitely reduced calcium absorption, which in turn may affect materially the mobilization of carbohydrates and other substances. The physico-chemical effects of ammonium may be expected to modify many metabolic processes such as hydrolytic reactions (hydrolysis of reserves, etc.), translocation of hydrolytic products, mobility and lability of sugars, amino acids, etc., respiration (in this investigation certain relations concerning dry weights seemed to suggest that respiration was little affected by ammonium nutrition under the condition of these experiments), and synthetic processes, which in turn largely determine the organo-chemical response of seedlings to ammonium nutrition.

In this nitrogen investigation of etiolated seedlings supplied with ammonium, injury resulted from the extreme deficiency of available carbohydrates. The concentration of ammonium did not increase greatly when carbohydrate deficiency became very severe; however, during this period leaching of nitrogen occurred from all species studied except perhaps the peanut seedlings. KULTZSCHER (14) concludes that the reaction of the cell sap is important in the storage of ammonium. More information is seriously needed concerning the extent and conditions under which ammonium may accumulate in plants without injury in order to more satisfactorily evaluate

the rôle of ammonium in plants. More extensive organo-chemical investigation supplemented with histological and physico-chemical studies are needed in order to obtain a more satisfactory conception of the conditions associated with so-called "ammonium injury" which in a large measure seems to be caused by the depletion of available carbohydrates.

### Summary and Conclusions

1. A brief resumé of PRIANISCHNIKOW's investigations pertaining to the rôle of ammonium in the nitrogen metabolism of etiolated seedlings and a critical analysis of his methods are included in the introduction.

2. With the adaptation of improved methods this investigation involves a further study of the effects of ammonium nutrition on the carbohydrate and nitrogen relations of etiolated seedlings. Seedlings of the following species were employed:

*Cucurbita pepo* (high oil reserve) pumpkin

*Arachis hypogaea* (high oil reserve) peanut

*Lupinus albus* (high hemicellulose and protein reserve) white lupine

*Lupinus luteus* (high protein reserve) yellow lupine

The seedlings were fractionated into stems, cotyledons, and roots for the analyses which were made at suitable intervals until the plants showed carbohydrate starvation symptoms.

3. In this investigation the conditions which resulted during ammonium nutrition cannot be ascribed to physiological acidity. In the light of modern principles of plant nutrition the methods employed by PRIANISCHNIKOW render the basis for his classification of seedlings unjustifiable.

4. The rate of absorption and utilization of ammonium was most rapid in the case of pumpkin. Although the oily reserve was rapidly converted into sugars no starch was formed therefrom. There was very little reversion of the sugars to fat in the seedlings. A marked depletion of sugars was associated with the rapid utilization of ammonium. Growth was favored by this association in the earlier stages during which time protein formation or regeneration was favored by ammonium nutrition. At the stage of extreme carbohydrate starvation (fourteen days) the protein and amide fractions were materially diminished in the stems while ammonium accumulated.

5. Although the food reserves of the peanut kernel are somewhat similar to those of the pumpkin, the response of the peanut seedlings to ammonium was very sluggish. During the early stages of germination and growth of the seedlings the oily reserve was rapidly converted into sugars, and in turn much of the sugar was converted into starch in the cotyledons and in the primary roots. At the time of carbohydrate starvation starch had entirely disappeared from the primary roots; however, a small amount of starch remained in the cotyledons. Even though the peanut kernels were high in oil



reserve the peanut seedlings behaved as physiological starchy legumes. The accumulation of amides favored by ammonium nutrition in the roots during the early stages was associated with a decrease in the sugars. Amides later decreased as sugars were further depleted. The ammonium peanut seedlings showed carbohydrate starvation symptoms at thirty days, while the controls showed similar symptoms at forty days.

6. Ammonium was utilized especially by the roots until the twelfth day in the case of the white lupine. Ammonium nutrition resulted in a marked depletion of amides in all organs during the carbohydrate starvation period (12 to 16 days) due to the exhaustion of the sugar supply resulting from earlier utilization of ammonium. Ammonium rapidly increased during the carbohydrate starvation period.

7. The yellow lupine seedlings utilized ammonium in the early stages (3 and 6 days). During the carbohydrate starvation period (6 to 9 days) ammonium was neither utilized nor absorbed. The amides were broken down and ammonium accumulated as a result of extreme depletion of available carbohydrates.

8. All the seedlings studied absorbed and utilized ammonium during early stages of germination while the synthetic processes were disrupted during the carbohydrate starvation periods when ammonium accumulated as a result of the breakdown of organic nitrogen because of the exhaustion of available carbohydrates. Ammonium injury appeared to be associated with carbohydrate depletion but is not ascribed to any specific condition.

9. In this investigation it appears to have been demonstrated that there are at least three distinct internal conditions with intergradations which are associated with the response of the etiolated seedlings to ammonium nutrition, namely:

(1) In the early stages of growth when available carbohydrates are abundant ammonium is readily absorbed and utilized resulting in protein synthesis which is especially evident in the roots. There is a tendency for growth of the seedlings to be favored by ammonium. The degree of response and duration of these early stages varies considerably with the various species and seems to be associated somewhat with the type and amount of food reserves stored in the kernel.

(2) In the intermediate stages when available carbohydrates are less plentiful, though ammonium is absorbed and utilized, proteins are broken down and amides accumulate. During this period ammonium has little effect upon growth.

(3) During the carbohydrate starvation period, when available carbohydrates have become seriously depleted, ammonium is neither utilized, nor absorbed; proteins continue to be broken down; amides are decomposed; ammonium accumulates, and there may, or may not be an increase in amino

nitrogen. There is a tendency for leaching of nitrogen from the seedlings to occur during this period. Carbohydrate starvation becomes progressively evident as characterized by cessation of growth followed by desiccation of the aerial organs. The accumulation of ammonium during this period is due to the breakdown of organic nitrogen compounds resulting from extreme deficiency of available carbohydrates.

UNIVERSITY OF CHICAGO

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# STRUCTURE OF THE STARCH GRANULE

C. L. ALSBERG

## Introduction

The purpose of this paper is to present an hypothesis regarding the anatomical<sup>1</sup> structure of natural starch granules. The hypothesis itself is not new; it is a combination of parts of older hypotheses, but with the introduction of some concepts new for starch, though not new in themselves. The hypothesis, reduced to its simplest terms, will first be stated without proofs at the risk of biasing the reader against it. The physical properties of granules, so far as pertinent, will be examined to determine whether the hypothesis is useful for understanding them.

The hypothesis is based on certain observations recorded in the literature or newly presented here. These observations are:

1. The starch granule may be made to swell and shrink by varying its water content.
2. At ordinary temperatures, swelling in water is limited; at higher temperatures, it is great and may lead to ultimate dispersion of most of the granule substance (gelatinization).
3. The granule, if mechanically injured, *i.e.*, if it is chipped or cracked, swells greatly in cool water at the site of injury, and only there.
4. The granule is insoluble in cold water.
5. The granule, if mechanically injured, is more or less soluble in cool water by partial dispersion of the swollen portion.
6. The natural granule consists—at least in large measure—of material in orderly arrangement and exhibits a characteristic x-ray diagram. The diagram may be changed by causing the granule to swell, and under some circumstances the process upon which the change in diagram depends is reversible.
7. Completely anhydrous starch exhibits no x-ray diagram.
8. Thoroughly ground-up and mechanically disintegrated starch exhibits no x-ray diagram.

## THE HYPOTHESIS

The peculiar anatomical organization of the intact granule restrains its swelling beyond a certain point in cool water. This restraining anatomical structure is the parallel arrangement of the long-chain macromolecules of which the starch granules' substance in large part consists. They are tied together in crystallites which are oriented radially in the granule. More-

<sup>1</sup> This word is used here to indicate that this paper does not deal with the finer molecular structure nor with the arrangement of the atoms in the molecule.

over, the starch-chain molecules possess an inherent tendency, probably due to intramolecular attraction of side groups for one another, to crumple or fold so as to reduce their own length. In crumpling, the macromolecule increases its diameter laterally; it thickens. In the natural granule containing adsorbed moisture, the macromolecules are not crumpled or shortened to their utmost because, being surrounded on all sides by other molecules which are also trying to thicken, room for complete contraction is lacking. The molecules at the same time endeavor to adsorb water, whereby they are also thickened. Thus, in swelling, a lateral pressure is exerted on the chains or bundles of chains, the micelles, which not merely prevents them from contracting but may even lengthen them so that the granule enlarges its diameter. In a sense, therefore, the macromolecules of a granule containing adsorbed moisture may be said to be stretched. They differ from the macromolecules in a mechanically stretched wool fiber in that the stretching force is a lateral pressure applied from all sides instead of a pull in the direction of the long axis of the fiber. The starch macromolecule is lengthened by squeezing, as one flattens a sheet of corrugated-iron roofing by lateral pressure; wool macromolecules are lengthened by pulling, as one straightens a coil of soft wire. The micelles are prevented from swelling to capacity, because there is not room for them to thicken as much as would be necessary if they sorbed or associated water to the maximum possible for them in a mechanically free state. Therefore, the swelling of granules in cool water is limited. Conversely, as a granule is dried, the transverse diameters of the macromolecules become smaller; the molecule shrinks laterally. The macromolecules crowd against one another with less force; space between them or between the micelles is made available to the extent that the water leaves. The granule shrinks until, in the completely anhydrous state, the chains approach a state of complete contraction.

So much for the hypothesis. We have now to present the evidence upon which it is based, and to test its usefulness in interpreting the physical properties of starch.

### Supporting evidence

#### SWELLING

It has been established conclusively by actual measurement that the starch granule shrinks when dried, and swells again when moistened (3, 64, p. 18; 77). MEYER (64) found that drying at 20° C. for only 2 hours shortened the longest diameter of a potato-starch granule, freshly prepared and washed in water, by 15 per cent., and this observation has been confirmed in the writer's laboratory. Further drying at 90° C. for half an hour reduced its diameter by only 4 per cent. more. Additional final drying at 110° C. failed to produce further shortening. Total shrinkage is clearly very con-



siderable and therefore difficult to explain, as we shall see, if starch is composed of chain macromolecules that behave like those of fibers.

As one would expect from the capacity of the granule to swell in cool water, granules saturated with moisture are quite elastic. They can be deformed by mechanical pressure; but their greatest possible linear increase is only 3 to 6 per cent. Though very elastic, they do not stretch much (70, p. 39).

Like textile fibers (8), starch seems to exhibit hysteresis with respect to moisture sorption. Only moderate shrinking is promptly reversible. Sharply dried granules, if exposed to water vapor, do not swell rapidly back again to their original volume (3, 107).

Moreover, even moderate drying diminishes the power of starch granules to swell when boiled. They do not—at least for some time—swell to the same size that they would have reached had they not been dried (3).

Drying raises the gelatinization temperature (56, 73, 76) and affects other properties as well.<sup>2</sup> The length of time starch has been wet also seems to be of influence upon the gelatinization temperature (81). If drying is carried to the extreme, but only then, the x-ray diagram of the granule disappears;

<sup>2</sup> The effect of treatment with alcohol and ether is probably also to be attributed to dehydration. Wheat starch treated only with ether yields pastes of much greater mobility than the same starch sample treated with both alcohol and ether (55).

Not merely does thorough drying alter the swelling and water-sorption capacity, but starch thoroughly dried is said to be more easily hydrolyzed than it is before drying (79). Possibly this is because drying tends to produce rifts and cracks. Injuries tend to cause some of the starch substance to disperse (see pp. 298, 318). This interpretation finds support in the observation of NÄGELI (70, pp. 52, 63, 107) that the rifts and cavities grow larger in dry potato starch when it is placed in water. MEYER (64, pp. 96–99) and BARANETZKY (13) have observed that dry grains are more quickly affected by diastase than freshly prepared ones, because fissures in them facilitate the entrance of the enzyme. MEYER also observed that potato starch, which is slowly attacked by diastase, is attacked more readily if the outer layer is broken.

BROWN and HERON (17) and MAQUENNE (59) have shown that mechanically injured granules are attacked with great rapidity by diastase, observations which have been confirmed by GRIFFING (*cf.* 2, 76).

These effects of drying are therefore probably due to rifting of some of the granules which renders them partially soluble (see p. 309, and *cf.* also 87, 88).

Dehydration may also account for some of the differences between root and seed starches. In roots, the starches are never dry; in many seeds, for example the small grains, the starch may be quite dry, for the moisture content may sink as low as about 6 per cent. Indeed, the condition of the starch may perhaps account for some of the peculiar properties of flour made from such very dry wheat. Clearly, the starch of wheat harvested with a moisture content of 18 per cent. is not in a condition identical with that of wheat harvested with a low moisture content, say 8 per cent. It may be that some of the granules of very dry wheat, such as is harvested in semi-arid countries like California and British India, are rifted and thus influence the diastatic power of flour made from them.

incompletely dried starch retains its x-ray diagram (47, 48, 65, p. 1111; 66), some starches losing their x-ray diagram more readily than others (47, 48).

It has long been known that the temperature at which the granules of a given starch species swell is distinctly different for granules of different size. The phenomenon has recently been studied in some detail by KATZ and HANSON (49). They concluded that each individual starch granule has a growth organization (*gewachsene Struktur*) which resists swelling and which must be overcome if gelatinization is to take place. No doubt there must be some such organization, but it may well be substantially the same in all granules of the same species and yet permit granules of different sizes to swell at different temperatures. Some years ago, the writer (3) pointed out that the relation of the mass of swelling material to the surface area of granules must in itself be a factor in determining the fact that in the same sample large granules tend to gelatinize more easily than small ones. The volume of a sphere or its mass increases as the diameter increases much faster than does its surface area. Therefore, given structure of identical rigidity in two granules of different diameter, the larger one should swell more readily than the smaller. It should swell sooner as the temperature is raised, because it will begin to exert enough intragranular pressure to distend the restraining structures of the granule before these have been softened to as great a degree as they must be to permit the smaller granules to swell at all. This explanation is supported by the fact apparently first pointed out by NÄGELI (70, p. 68) that in granules with very eccentric lamination swelling begins sooner on their eccentrically larger side, while the smaller side is still unchanged.<sup>3</sup> Indeed in very eccentric granules swollen in dilute potassium hydroxide the eccentrically larger side may swell around the still unswollen hilum end so that the latter forms a depression until it, too, swells and the whole granule becomes more or less uniformly inflated (70, p. 77). The pressure exerted in swelling, however, must be unequal in such granules on the two sides, for the reason given above. It must be greater on the larger side and swelling must be greater there than on the smaller side.

#### MECHANICAL INJURY

That mechanically injured granules swell in a different manner was first observed by SCHLEIDEN (84), and has often been confirmed. A cracked or chipped granule swells at the site of injury and only there. HARRISON (36) has reproduced a photomicrograph of such a granule. Injured granules,

<sup>3</sup> KATZ and HANSON (49), however, report that in every sample there are a few granules which partially resist gelatinization when heated to the threshold temperature at which gelatinization begins. Only a part of them becomes isotropic and stains with Congo red. This is commonest for eccentric granules. The eccentrically larger side may remain ungelatinized even when the smaller side has already undergone this change.

however, do not swell in anhydrous liquids such as pure glycerol; though in reagents like potassium hydroxide swelling begins at the injured spot (11). In water, swelling does not extend into the interior of the granule and then gradually throughout the whole of the granule as one would expect if a limiting membrane were the only mechanism that prevented the swelling of uninjured granules. Nor does the swelling spread circumferentially to involve the material between the laminae that have been fractured, as one would expect if the laminae were separated by membranes. These phenomena are particularly striking in granules broken in two by a fracture passing through the hilum. The two halves swell and disperse only over the surface of the fracture and for a short distance beneath. The halves do not swell *en masse*. They retain a normal appearance and their optical properties do not change except at the swollen fractured-surface region (31). HUSS (41), moreover, has demonstrated that triturated potato starch suspended in water occupies a greater volume than an equal weight of uninjured starch because a part of it is swollen. Indeed, the degree of swelling depends upon the extent of the injury, for the swollen portions do not wholly disperse. An extensively injured granule may swell to very nearly the size it would have reached if boiled in the uninjured state, as was established by measurement with a micrometer eyepiece (31). This is apparently because the natural granule contains  $\alpha$ -amylose (amylopectin),<sup>4</sup> which swells in cold water but does not disperse.

<sup>4</sup> There is much confusion in the nomenclature. It seems generally agreed that all starches contain a substance easily dispersed in water. For it, MEYER (64) introduced the term,  $\beta$ -amylose, which is still very generally used, though sometimes this substance is simply termed amylose.

Many starches also contain insoluble material, which MEYER termed  $\alpha$ -amylose, but later MAQUENNE termed it amylopectin. At that time, it was not yet known that some starches contain combined fatty acid of high molecular weight, while others contain none but usually instead phosphoric acid. The presence of phosphoric acid was discovered first, and it was believed that its presence was characteristic of  $\alpha$ -amylose or amylopectin, so-called. SCHOCH (85, 105), however, has shown that in potato starch there is no insoluble fraction. According to this view, amylopectin is not a chemical individual, but merely  $\beta$ -amylose esterified to different degrees with phosphoric acid, or  $\beta$ -amylose in different degrees of association esterified with phosphoric acid. KADEN (44), working under the direction of C. J. LINTNER, has questioned its very existence. Moreover, some native starches apparently contain no insoluble material at all, but consist wholly of  $\beta$ -amylose (38).

The fatty-acid compound occurs preformed in cereal starches; it is insoluble. It is the only insoluble substance whose existence preformed in native starches has not been questioned. It follows that it is the only substance generally admitted to occur in native granules which conforms to the specifications as regards insolubility laid down for amylopectin by MAQUENNE, who introduced this term.

It seems, therefore, inadvisable to continue to employ the term amylopectin, for not all starches contain preformed insoluble material, though they may contain combined phosphoric acid. Further, amylopectin, so-called, has none of the chemical characteristics

The swelling of injured granules seems to be quite analogous in character to that of granules gelatinized by heat. The two sorts of swollen granules behave similarly with respect to staining and they exhibit similar optical properties. Tannin acts only upon that part of the granule which is injured in the same manner in which it acts upon boiled granules (14). Intact granules do not stain with such dyes as Congo red (54), which are among the colloidal dyes, though granules are stained by other dyes. FISCHER (27) found that, in granules impregnated with these dyes, picric acid precipitates the dyes in crystalline needles arranged radially. This may be taken as evidence of radial structure of the granule.

Injured granules, however, stain easily with the dyes, like Congo red, that do not stain intact granules, the staining being restricted to the injured part (27, 31, 41, 112). Heat-gelatinized granules stain like the swollen, injured portions of unheated granules, but the staining tinges the whole of the granule (30). HUSS (41) has, indeed, proposed the use of this phenomenon as an indicator to fix gelatinization temperatures. Congo red stains only the swollen parts of granules; allowing the granule to remain in the dye solution for 48 hours does not result in the penetration of the dye much, if at all, beyond the swollen area. The dyed material appears granular, taking the dye unevenly, whereas granules gelatinized by heating stain uniformly and appear more homogeneous.

Furthermore, Congo red dissolved in anhydrous glycerol does not stain injured granules, nor does it stain whole granules. After standing some time, however, the glycerol takes up water from the atmosphere and the injured granules begin to swell.<sup>5</sup>

The swollen portion of an injured granule loses its birefringence exactly like a granule gelatinized by heat. Here it should be pointed out that KATZ and HANSON (50) believe that wet grinding as employed by ZWICKER and by them acts in a way different from dry grinding as employed by SPONSLER (95) and by ALSBERG and PERRY (7). KATZ and HANSON found that wet grinding does not destroy birefringence (black cross with nicols crossed) or the x-ray diagram. As we shall see, dry grinding does not destroy birefringence either, except in the injured portion. In starch ground wet, change

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of pectin, nor does it serve similar functions. It has seemed best, therefore, to continue the practice of the writer and his co-workers in following TAYLOR and his co-workers (102-106) and SHERMAN and his co-workers by employing the term  $\alpha$ -amylose for the insoluble fraction, and the term  $\beta$ -amylose for the soluble fraction. It is recognized that this practice is open to the objection that the Greek-letter prefixes have no essential connection with a stereochemical difference of the units composing the starch constituents, and that KATZ and WEDINGER (53) have questioned the identity of TAYLOR's substances with the correspondingly named ones of MEYER.

<sup>5</sup> According to FISCHER (27, p. 71), air-dry granules swell appreciably in a mixture of equal volumes of anhydrous glycerol and water.

in birefringence in the injured portion is not manifest because the injured portion swells and mostly disperses (*cf.* pp. 298–299). According to SPONSLER, sufficiently severe dry grinding destroys the x-ray diagram. According to KATZ and HANSON (*cf.* also 51), wet grinding does not. This difference is probably due not to difference in mode of action of the two methods of grinding, but to the fact that wet grinding, as employed by KATZ and HANSON, did not disintegrate the granules nearly as completely as the very long dry grinding employed by SPONSLER. Starch subjected to a shearing pressure (*gleitendem Druck*) of 20,000 atmospheres loses its x-ray diagram (65).

The behavior of the swollen, injured part of a granule is similar to that of a granule swollen *in toto* by heating in water. Everything indicates that the swelling of injured granules is brought about by the same mechanism as gelatinization by heat. This suggests that neither gelatinization nor the alteration of the x-ray diagram brought about by gelatinization as described by KATZ and co-workers<sup>6</sup> is accompanied by chemical alteration of the molecule in the strict meaning of the word.

#### MEMBRANES

Many investigators (65) assume the existence of an outer, limiting membrane to explain some of the phenomena above described. In fact, there seems to be an outer, visible membrane, probably part of the protoplast which according to HANSON and KATZ (34), may be more or less lost in the process of preparation. According to ZWIKKER (112, pp. 61, 68), the plastid is removed in the preparation of starch. It is obviously a delicate structure. Perhaps it affects some of the physical properties of starch. Thus LACHELE (55) found that the granules of a commercial sample of wheat starch (Merck's), although neutral in reaction, became ruptured when swollen by boiling in water for 20 minutes, whereas a sample prepared by the method of RASK and ALSBERG (75), which employs no other reagents than 1 per cent. sodium chloride solution, water, alcohol, and ether, did not. Naturally, the plasticities of the two starch preparations were different. Possibly the difference in their behavior was due to the removal in the one case and the preservation in the other of a natural, outer membrane or coating. In any event, such an outer coating can be but little concerned in the phenomena under consideration. If it were responsible for them, then commercial starches which contain granules that have lost this membrane in whole or in part (*cf.* 34) would contain an appreciable number of granules which are easily stained and disperse in water. As a matter of fact, such starches contain few such granules, and these are always plainly injured ones.

<sup>6</sup> See series of papers beginning in 1930: *Abhandlungen zur physikalischen Chemie der Stärke und der Brotbereitung*. In *Zeitschrift für physikalische Chemie*. Abteilung A.

THE MEMBRANE IN GELATINIZATION.—The view that natural granules have a strong outer membrane is based not upon seeing such a membrane with the microscope (*cf.* 64, p. 149) in unaltered granules, but upon observations on gelatinized granules. These observations are of two sorts: the appearance of gelatinized starch, and the distribution of phosphorus chemically determined.

Under the microscope, gelatinized granules appear to be sacs with elastic walls enclosing sol or very dilute gel.<sup>7</sup> The existence of a membrane could be demonstrated with a dissecting microscope, the microneedles of which had been replaced by fine glass hooks. These hooks were made to puncture a sac at opposite poles. By gradually screwing the hooks apart, the sacs of potato starch could be stretched to about twice their length. If stretching is continued much beyond this point, the sac ruptures suddenly as if made of over-stretched elastic. If the stretched sac is not ruptured and the strain is then relieved, it resumes its original shape. By means of a resistance wire, the paste was heated on the stage of the microscope and the manipulations repeated. It seemed a little easier to deform the warm granules, but the difference was not great. It was not possible to decide whether cold, gelatinized granules contain gel and hot ones sol (32).<sup>8</sup> There can be no doubt, then, that gelatinized granules are surrounded by an elastic, limiting membrane. It is this fact, principally, that has led to the inference, erroneous as we shall see, that this membrane preexists in natural granules.

PHOSPHORIC-ACID CONTENT OF MEMBRANE.—The membrane of gelatinized granules has been reported to contain phosphoric acid and has been assumed to consist of amylopectin which many investigators identify with the insoluble material of the granule. However, some starches consist wholly of  $\beta$ -amylose (*cf.* 38).<sup>9</sup> The belief that amylopectin is the insoluble material of starch and

<sup>7</sup> That this is the structure of gelatinized starch is also shown by the fact that the consistency of starch paste depends upon the maintenance of this structure (*cf.* 3, 34). Paste loses plasticity if the swollen granules are ruptured. Grinding in a pebble mill, for example, converts paste into a more or less viscous liquid, because the sacs collapse, thus no longer occupying nearly all the space of the system (7).

<sup>8</sup> Analogous observations were recorded by HESS and RABINOWITSCH (39), who examined granules that had first been gelatinized in water and then put into dilute alcohol. This may account for some of the minor differences between their observations and those of GRIFFING.

<sup>9</sup> In this connection, it is worthy of note that in the only starch (rice) in which the ash of the glutinous and non-glutinous varieties was compared, the non-glutinous variety had, on the average, more phosphoric acid and silicate than the glutinous variety. But some samples of the latter contained more of both than some samples of the former (101). The glutinous variety moreover, had the greater swelling power and yielded the more viscous pastes in spite of its generally lesser phosphoric acid content, a fact which does not support the view held by SAMEC and others that viscosity depends principally upon the phosphoric acid-containing fraction.

that it forms the membrane of gelatinized granules has led many investigators<sup>10</sup> to assume that the peripheral zone of ungelatinized granules also consists principally of  $\alpha$ -amylose (amylopectin). A difference in the phosphoric acid content of large and small granules of the same species has been advanced as evidence for this view. The reasoning is as follows: If  $\alpha$ -amylose is localized principally at the surface of granules, small granules of the same sample should contain a larger percentage of phosphoric acid than large ones, assuming the thickness of the membrane to be about the same in all granules, because the ratio of surface to mass is greater in small than in large granules. Small ones have a larger surface, relatively, and therefore a larger percentage of their mass should be in the membrane, if this is not thinner than that of large granules. SCHOEN (86), however, would attribute any greater phosphoric acid content of small grains to sorption on their greater surface. HINES made an investigation under the writer's direction upon cassava starch, separated into fractions of different granule size by air flotation in an air classifier, and failed to find any correlation between granule size and phosphoric acid content (1). It is therefore clear that there are starches for which no conclusion regarding the distribution of amylopectin can be drawn from their phosphoric acid content.

Furthermore, evidence controverting the view that small granules contain more phosphorus than large ones is to be found in the behavior of granules when boiled in water. If  $\alpha$ -amylose (amylopectin) were localized at the periphery, one would expect those natural granules to have the thickest and strongest membranes that have the greatest phosphoric-acid content. One would expect such granules when boiled to hold together better than granules with a lesser phosphoric-acid content. As a matter of fact, there is no such relationship between phosphoric-acid content and resistance to boiling. Potato starch with a fairly high phosphoric-acid content disintegrates rapidly, whereas tapioca, also a root starch but with a much lesser phosphoric-acid content (106), does not (31).

Moreover, MAQUENNE and ROUX (60), who first estimated the amylopectin content of granules, believed that it is intimately mixed with the other constituents. SCHOCH (85), in his study of the distribution of phosphoric acid, reached the conclusion that phosphoric acid is quite irregularly distributed through the natural granule.

<sup>10</sup> For example, (66, p. 216). LING and NANJI (57), however, hold a different view. They suggest "... that 'amylose' as it exists in starch granules is present in more than one physical state and possibly in different degrees of hydration ... about 25% of the 'amylose' exists as a crystalloidal phase in the form of spherites constituting a core round the hilum of the granule. This portion is readily extracted by water or by dilute alkali. The remainder, which presents a colloidal phase, is dispersed uniformly in the amylopectin layers. It appears to exist either as a solid solution or to be so strongly adsorbed on the amylopectin as to resist extraction.'

THE MEMBRANE AN ARTIFACT.—In summary, it may be said that there is no doubt that gelatinized granules are surrounded by a fairly strong membrane. The evidence that in ungelatinized granules amylopectin is concentrated in a peripheral membrane is doubtful. This, together with the absence from natural granules of a visible membrane of the thickness and toughness of the membrane of gelatinized granules, leaves the question of the existence of such a membrane open.

But the facts regarding swelling, shrinking, and staining, above arrayed, cannot be reconciled with the assumption that natural granules are surrounded by a membrane which is impermeable to many dyes, and to the starch substance itself. If the resistance of intact granules to staining and to more than moderate swelling and their insolubility in cold water were due merely to the existence of such a simple membrane, then in an injured granule with a locally ruptured membrane staining should be general throughout the granule and swelling in cold water should be general also. As we have seen, none of these phenomena occur. They are strictly localized to the site of the injury. As we shall see later, the birefringence of granules, as well as their solubility, is affected in an analogous way.

It is conceivable that the granule is surrounded by a membrane so rigid that it prevents mechanical disorganization except at the site of the injury where the membrane is broken. This is, however, improbable, because if there were a rigid membrane permeable to water this should either prevent shrinking or else one should be able to observe a separation of the shrunken granule substance away from the rigid membrane. It is improbable that a rigid membrane and the granule substance both shrink to exactly the same degree. Certainly in a dried granule no separation between membrane and granule substance is to be seen. If there is a limiting membrane which is elastic, like the membrane surrounding a gelatinized granule, it could hardly account for the strict localization of the swelling to the site of injury.

Some investigators have assumed the existence of a simple system of membranes represented perhaps by the laminae or rings. If there is such a system, the laminae are hardly a part of it, for unlaminated starch swells like ordinary starch (12). Moreover, a granule broken in two by a fracture passing through the hilum has all of its rings opened up. Any simple membrane system that might be present would be opened up widely so as to permit extensive, if not complete, swelling and staining. As we have seen, these phenomena are strictly limited to the immediate region of the fracture. Certainly, a series of concentric membrane rings could not explain the facts. Nor could a series of radial membranes. Nothing short of a system consisting of concentric, hollow spheres, divided by many radially arranged transverse planes could explain the behavior of fractured granules.



KATZ and RIENTSMA (52) have, indeed, suggested this very possibility. KATZ (46) subsequently elaborated this idea as follows: "There might be a binding substance in a swelling substance which binds packages of micellae together to larger, probably, but still submicroscopical units. In grown products, tissues, fibers, starch granules, this binding substance might take the form of membranes lying between the micellae or between small bundles of micellae."

Subsequently, HANSON and KATZ (34) obtained support for this view by the examination of Lintner starch,<sup>11</sup> which easily fragments into concentric rings composed of separate rectangular blocks regularly arranged radially. These blocks they believe to be composed of bundles of parallel crystals, for they are normally birefringent. They believe further that the blocks are held together in natural starch by a cementing substance, possibly amylopectin ( $\alpha$ -amylose), which is dissolved out by the acid in the process of lintnerization. Lintner starch does not swell in cold water, but disperses to a clear solution in warm water. It does not form paste because the cementing substance which holds the granule together and swells in the process of gelatinization has been removed. It is the cementing substance, they suggest, that forms the membrane system of the natural granule.

It is altogether likely that the view that there is intermicellar substance is correct,<sup>12</sup> but it may be doubted that it serves as a membrane system, for its presence does not seem capable of explaining the swelling of injured natural granules. Why should the cement substance swell so little in the cold in an intact granule and so greatly in an injured one? Obviously, some additional factor is involved.

On the other hand, if the natural starch granule swells greatly in water after it has been broken up by mechanical means, why does it not swell when it is broken up into blocks by Lintner's treatment? It must be either that the prolonged acid treatment so greatly modifies the granule's substance that its swelling power and solubility are radically altered, or else that, of the substances of which the granule is composed, only one, the cementing substance, swells and disperses cold. The Lintner process may remove this cementing substance.<sup>13</sup> In that event, the other material, composing the blocks of Lintner starch, neither swells nor disperses in water, except when warmed. Thus we might explain the properties of Lintner starch; but we would not explain why natural intact granules which contain the swelling substance do not swell greatly unless warmed, whereas the same granules, still containing the swelling substance, swell greatly at once when injured.

<sup>11</sup> Starch subjected for a long time to 7.5 per cent. hydrochloric acid.

<sup>12</sup> LOSKIT (58, p. 154) concluded from the optical properties of starch that it must be a mixture of colloidal and crystalline particles.

<sup>13</sup> Commercial soluble starch and the artificial starch granules of MAQUENNE and ROUX do not yield amylopectin (33).

We are thus forced to the conclusion that such membrane as natural granules may possess is not identical with the limiting membrane of heat-gelatinized granules. How is this to be explained? Obviously, by the assumption that the membrane surrounding heat-gelatinized granules is an artifact. ZWIKKER (112) has demonstrated that this is true. He has correctly described the origin of the membrane during gelatinization thus: "The outer parts of the grain become distended; they detach themselves at the same time from the central mass or remain adherent to it. In one case as in the other, a cavity is formed in the interior of the grain. The warm water or any other liquid that may have been used to make the starch swell is thus aspirated so to say into the interior. In passing through this wall it [the liquid] removes from it [the wall] and carries along with it [the liquid] the dispersible substances; in consequence, when the gelatinization has become complete, the membrane is only composed of the elements that are soluble with difficulty derived from the different parts of the grain and especially from the most resistant layers; all these elements are found agglutinated into a homogeneous mass."<sup>14</sup>

That granules in process of being gelatinized by heat do show a central cavity which gradually enlarges, has also been observed by the writer and by many others (64).<sup>15</sup> Very commonly, this cavity is at first stellate, due to the radial structure of the granule. The observations of BAKHUYZEN (12) upon wheat starch devoid of laminae support the view of ZWIKKER. There is, therefore, no necessity to assume the pre-existence in natural starch of a rigid, limiting, outer membrane. The gelatinized granule's membrane is clearly an artifact.

On the assumption that natural granules possess no limiting membrane, the behavior of compound granules, when gelatinized, becomes understandable. After they have fallen apart into their component granules each of the latter forms its own limiting membrane when gelatinized, exactly like a simple granule. And this occurs even when no membrane separating the component granules is visible in the original unbroken compound granule.

Furthermore, occasionally two granules that lie close to one another in the same protoplast become enveloped by a capsule of starch substance which shuts them off completely from their surroundings. MEYER terms them complex granules. When they are gelatinized by heating in water the whole complex is connected into a sphere with only one outer limiting membrane (70, pp. 210, 224). If the two granules had each its own outer membrane, one would expect the complex granule to be changed into a large sac surrounding two smaller sacs—one for each of the component granules. Their

<sup>14</sup> The writer's translation from the French original.

<sup>15</sup> A drawing of a granule in this state is given by HANSON and KATZ (34).

behavior when gelatinized is easy to explain on ZWIKKER's hypothesis but difficult on any other.

#### UNLAMINATED STARCH

The facts presented seem to warrant the conclusion that it is not a membrane system that keeps the natural granule from swelling beyond definite limits. If this is so, what is the mechanism that is responsible for this phenomenon? The hypothesis advanced in this paper presents such a mechanism: it is the radial arrangement of the crystallites and starch chains. To make clear how this simple physical arrangement explains the facts, it is best first to consider granules without laminations or rings. This is justifiable because the occurrence of laminations is accidental, rather than essential, otherwise they would never be lacking in any sort of starch. But they are occasionally missing from many sorts of starch and their absence is the rule in the root starch of *Zingiber officinale* (70, p. 17), in the starch of the yellow turnip, and the seed of *Ruellia pavale* (109). MEYER (64) attributed their formation to the alternation of day and night; moreover, BAKHUYZEN (12) failed to find them in seed starch from wheat grown in an environment from which this sort of periodicity had been eliminated—that is, an environment nearly constant with respect to illumination, temperature, and humidity.

BAKHUYZEN's un laminated wheat starch has very definite radial structure. In it, the crystallites may be seen to reach from the periphery to the hilum. When these granules were carefully heated in water so that they swelled slowly, he found that they could be seen to be composed of very refractive radial needles attached to the refractive border of the granule. These needles are tapered toward the center, resembling pyramids with a base 2 to 3 microns in diameter and a length of 17 to 20 microns, which is about 45 per cent. of the total diameter of the granule. In such unheated, un laminated starch, probably all of the needles (crystallites) stretch from the border to the hilum.

#### THE STARCH ELEMENTS

X-ray analysis has definitely established that the starch granule has crystalline structure. Most probably the ultimate starch elements are long units (45, 98, p. 75).<sup>16</sup> It is not yet possible to say just what is their form, but it is probably not a straight chain, for since the elements of the chain are apparently held together by  $\alpha$ -glucosidic linkages, a straight-chain structure is not possible (37). Perhaps starch chains are spirally wound (65),<sup>17</sup> like the thread on the end of a bolt that receives the nut, or have a zigzag (65) shape

<sup>16</sup> See, however, (19).

<sup>17</sup> It is interesting to note that spirally twisted granules have been reported by NÄGELI (70, p. 31).

like the bellows of an accordion, or a meander (99) form, being fluted more or less like a sheet of corrugated roofing iron. In any event, the long chains seem to be folded or crumpled in some manner as yet unknown, whereby the molecule is shortened—compressed as it were—in the direction of its long axis, for a starch molecule consists of 8 to 10 times as many glucose residues as a cellulose molecule of the same length (99, p. 828). Perhaps the chains are crumpled and the long axis of the molecule shortened because certain of the side groups of the macromolecule attract each other strongly. This would tend to bend the long axis and shorten the molecule. It is possible the chain is also forked or branched (66, 99). Units of this type would not pack as closely as straighter chains, for example, those of cellulose. This may be one of the reasons why starch has a looser structure than cellulose, is more easily penetrated by solvents (99), and is more readily subject to mechanical influences (45, 95).

In these long units, the atoms are linked together by primary valences. It is probable that van-der-Waal or cohesive forces hold these macromolecules together in more or less parallel arrangement. Whether in this way the macromolecules are held together in separate and discrete bundles to form micelles is not clear. At present, the view seems to be gaining ground that the micelle, in general, is not always and necessarily a separate and discrete unit and that fibrous structures like cellulose need not have a completely discontinuous framework. NEALE (72, 89) has suggested that in certain cases, at least, the structure is not "micellar" or discontinuous, but that, though imperfectly crystalline, it is continuous except for random breaks in the primary valence chains and that though continuous it is slightly imperfect. TAYLOR and SCHOCH (105) have suggested the presence in starch "of more or less completely esterified tri-basic phosphoric acid, either with hydroxyl groups on one chain or bridging between two chains of glucose anhydrides."

#### GRANULE STRUCTURE

Everything indicates that the chains, or the bundles (micelles) into which the chains are perhaps grouped, are arranged radially.<sup>18</sup> Of course, the radial granule structure visible with the microscope is a coarser one than that of the macromolecules or even the micelles. Each of the visible radial needles must be regarded as a crystallite composed of many chain molecules or of many micelles of such molecules.<sup>19</sup>

A granule thus constructed would be strongest in a radial direction. The primary valence bonds which hold the glucose residues into a chain would

<sup>18</sup> This is similar to the anatomical structure proposed by MEYER (64). The bundles would correspond to his trichites, but he assumed the trichites to be forked.

<sup>19</sup> Whether the macromolecules are arranged parallel in the crystals has not yet been proved by x-ray analysis, but is probable according to MEYER, HOPFF, and MARK (65).

make for greater strength lengthwise of the chain than would the secondary valences which hold the chains to one another laterally (94). In consequence, there is a tendency for granules to rift radially when subjected to mechanical strain. The force required need not be very great; as we have seen, mere thorough drying may cause radial cracks to appear. This fragility need not be taken to mean that the structure of the granule is everywhere discontinuous from micelle to micelle or that chemical cross linkages between chains are necessarily absent.

Does this suggested structure explain the shrinking and limited swelling of natural granules?

Let us first consider the shrinking of granules when dried. If the starch units are long chains, then when they swell they must increase in diameter much more crosswise than lengthwise, if indeed they lengthen longitudinally at all (96).

And conversely, they must diminish more in their transverse diameter than longitudinally when they shrink. This seems to be the usual behavior of fibers. The diameter of the wool fiber becomes about 18 per cent. greater when it is wetted, but only 1 per cent. longer (8). Therefore, it is difficult to explain the very considerable shrinkage of starch granules when they are dried (78), for the radial arrangement and the swelling and shrinking of long chains in their shorter diameter should result in swelling or shrinking in a tangential direction. As a consequence, the shrinking granule should break up into wedge-shaped pieces. This is actually the behavior of inulin spheruliths (27) and of the crystalline disks of amylopectin (69). It is probably also a factor in the radial rifting of dried starch already noted. Since, however, the starch granule swells and shrinks in a radial as well as a tangential direction and increases or decreases its diameter, we must assume either that the starch chains do not behave like wool fibers or else that for some reason they are prevented from so behaving.

This difficulty disappears if we assume the starch chains in the natural moist granule to be already stretched mechanically by their own swelling pressure. As we have seen, according to STAUDINGER and EILERS (99), the starch chain is shortened in some way, as compared with cellulose, a conclusion based in large part upon viscosity determinations of starch solutions.<sup>20</sup> In solution, the macromolecule is under little or no mechanical strain. It is, therefore, presumably much crumpled so that its long axis is much shortened. It may, however, not be maximally contracted. There is no basis for knowing whether starch macromolecules have the same length in solution as in the natural starch granule.

<sup>20</sup> This interpretation of the significance of viscosity measurements has, however, been questioned (18, 62).

It is obvious that at maximum contraction the region over which water or other solvent might associate itself with the macromolecule is shorter than when the macromolecule is more or less extended. In the contracted state, secondary valences are in part neutralized by the very intramolecular attraction that shortens the chain. Moisture sorbed on natural starch granules is under quite enormous pressure (77) and we are, therefore, justified in assuming that water exerts very great force in attempting to associate itself with starch. In doing so, water would endeavor to make as much room for itself along the length of the chain as possible. To this extent, the forces crumpling the chain, such, perhaps, as the intramolecular attraction of side chains for one another, would be counteracted and weakened. The chain would tend to straighten. It is, therefore, probable that starch molecules, while shorter than cellulose molecules though with the same number of glucose residues, are still, as STAUDINGER and EILERS believe, quite long chains when in solution. In this condition, they are neither fully contracted nor fully extended. When a starch solution is dried to a film, there may well be further contraction of the macromolecules.

If these conceptions correspond to realities, it follows that, in natural moisture-containing starch granules, the chains are extended to the degree that associated water has stretched them. But their state of extension is probably not the same as in a starch solution. Presumably, as in the case of wool, the association of water tends to thicken the diameter of the chain. We would, then, have two factors at work in opposite directions: lengthening of the chain which would tend to make it thinner, and the pressure of associated water which would tend to make it thicker.

When a cellulose or a wool fibril sorbs water, it increases greatly in its transverse axis and but slightly along its longitudinal axis. Such a fibril is free to expand in all directions except inward where it is hindered by other fibers also swelling similarly, and, therefore, increases greatly in diameter. A starch fibril is in a quite different position. Because of the radial arrangement of the granule, it is surrounded on all sides of its long axis by other fibrils also endeavoring to swell in a direction transverse to their long axes. Every macromolecule is prevented from associating itself with water to its maximum capacity because it is hampered mechanically by its position with reference to its neighboring macromolecules. That is why the intact starch granule is strictly limited in its capacity to swell despite the fact that its substance has unlimited capacity to swell and disperse. No limiting membrane is necessary to account for the phenomenon.

According to our hypothesis, the macromolecules in moist natural starch are perhaps unable to associate themselves with as much water as is possible when they are free in solution. Nevertheless they may be more extended along their long axis when *in situ* in the moist granule than when free in solution.

for in the granule the macromolecules squeeze one another laterally; they subject one another to strain. In consequence, they tend to push one another apart and, since this is possible only to a slight degree, they flatten and lengthen one another.

The possibilities for such lengthening are appreciable, since the chains tend, if not subjected to strain, to assume a very crumpled form. The dry granule, when moistened, tends to swell and its thickness is a function of the moisture content. Any condition such as heating that changes the moisture content, *i.e.*, the association or sorption of water, changes the size of the granule; it shrinks or swells. In the natural moisture-containing granule, the chains are already stretched to some degree due to their own swelling pressure. That is why the granule shrinks when dried and swells again when moistened.

It is also the reason why the moist granule does not swell beyond a certain size. It could do so only if there were room for the macromolecules (or micelles) to thicken more; but there is no room because they are surrounded on all sides, except at the peripheral end, by other macromolecules or micelles which are also trying to thicken. Swelling, therefore, stops when equilibrium is reached between the force making for thickening and the resistance to thickening.

This equilibrium may be disturbed by increasing the kinetic energy of water as by heat. Molecular agitation which is associated directly with the coefficient of thermal expansion would be less lengthwise than crosswise of the starch chains (*cf.* 97) and therefore granules tend to swell more in warm than in cold water. Greater swelling in warm water can take place only if the diameter of the granule enlarges. Since the macromolecules (or micelles) swell mostly laterally, this can take place only in the following ways: (1) The macromolecules straighten their long axes by water sorption and pressure against one another. (2) The macromolecules push one another apart, though still cohering. In consequence, the diameter of the granule increases greatly so that spherical granules are converted from solid spheres into hollow globes. (3) The granule is disorganized and more or less dispersed. (4) Or, there is a combination of these phenomena.

The first phenomenon, the straightening of the macromolecules accompanied by water sorption, probably occurs in the early stages of gelatinization and may perhaps account for changes in the x-ray diagram.

The second phenomenon, the conversion of a solid spherical granule into a hollow sphere, may actually be observed when the granule is slowly heated in water. It swells and, as we have seen, a cavity develops at the hilum, because the degree to which the chains may lengthen must be strictly limited. The granule cannot swell greatly and still retain its solid form. Spherical granules become hollow spheres. Other granules swell in a way determined

by their form and the fact that swelling develops pressure tangentially. Thus, it is well known that eccentric granules (*Solanum*, *Phajus*, cassava, *et al.*) swell relatively more in breadth than in length.

KATZ and HANSON (50) have assumed that the swelling of solid granules to hollow spheres is a unique phenomenon, and that the only possible way to explain it is to assume some sort of growth structure in the native granule. They have not themselves determined the nature of this structure resulting from growth. Protein crystals, however, also swell with the formation of a vacuole in the interior, and may be made artificially. SCHIMPER (83) attributed the phenomenon to the greater solubility of the material in the interior of the crystal, and this is the explanation NÄGELI had offered earlier for the analogous phenomenon in starch. However, the explanation offered by ZWIKKER and by BAKHUYZEN for starch seems equally applicable to protein crystals, if one assumes that the outer layers swell soonest and tend to draw away from the center, thus creating a vacuole. It is then unnecessary to assume, as did SCHIMPER, that these crystals consist of more than one protein, which would not be in accord with present-day knowledge. Nor is it in accord with the fact that WYCKOFF and co-workers have obtained x-ray diagrams from protein crystals. Some protein crystals behave so much like starch with respect to swelling and solubility that if one has a "growth structure" then they both have. This "growth structure" is the arrangement of their molecules in the crystal. The hypothesis here presented, should it be supported by further experimental evidence, offers a simple and reasonable explanation of the phenomena.

With heating, the granule swells greatly. The second phenomenon, disintegration, has then occurred; the granule has been converted into a sac filled with dispersed starch substance; and we have the third phenomenon. The degree to which disorganization occurs is a function of the amount of water available, as has been shown by MEYER (63).

If this hypothesis should ultimately prove to be well founded, then analogies with rubber at once come to mind. The moist starch granule is elastic; it changes its shape under pressure and returns to its original form without the slightest change in its microscopic structure. Dry granules, on the contrary, are inelastic and brittle. Rubber, too, under certain conditions, loses its elasticity. Moreover, it is well known that rubber exhibits the x-ray diagram of amorphous substances unless it be stretched, in which case it exhibits a fiber x-ray diagram. When it is quite anhydrous, starch, like unstretched rubber, exhibits no x-ray diagram, or rather it exhibits the diagram of an amorphous substance (45). It acquires an x-ray diagram only if it is permitted to sorb moisture, but the diagram is not that of a fiber. It is more like that of a crystalline powder. According to our hypothesis, we may consider the macromolecule of a natural undried granule as already



partially stretched by associated water, very small quantities of which are capable of stretching the chains to give them an orderly arrangement sufficient for an x-ray diagram.

Hitherto it has not been possible to obtain a fiber x-ray diagram from starch. Neither spinning threads from starch solutions nor pressing preparations was effective (65). For natural starch granules, a fiber x-ray diagram is not to be expected. For a fiber diagram, it is necessary that the chain molecules of the material be oriented so that their long axes for the most part are parallel. In a powder consisting of starch granules, this is not the case. Nor would a single starch granule yield a fiber diagram, if it were possible to obtain an x-ray diagram with it. Although the macromolecules are probably parallel in the individual crystallites, the crystallites themselves are not parallel to one another. Their arrangement is radial. Therefore, although in the moist granule the macromolecules may be regarded as more or less stretched by their own swelling pressure, they cannot yield a fiber diagram.

NÁRAY-SZABÓ (71, p. 299) and KATZ and DERKSEN (48) have studied the loss of the x-ray diagram in quite anhydrous starch. KATZ (45, pp. 3633–3635) has also described a similar behavior for gelatin and agar, and has offered the following alternative explanations: One of them is that the substance when dried changes into another modification, another phase within the meaning of the phase rule. This means that the material, *e.g.*, agar gel, is built up of crystallites which contain water of constitution. The other alternative is that in drying no change of phase occurs (within the meaning of the phase rule) involving a discontinuous modification of individual units, but rather there is a continuous change involving a gradual loss of regularity in the lattice. KATZ makes no choice between these alternatives in the case of gelatin and of agar, but in the case of starch he is inclined to believe that there may be something like true water of crystallization. Perhaps he favors this view because all starch preparations (soluble starch, amylopectin, etc.) exhibit the same phenomenon (45). What is meant by “water of crystallization” is, of course, a matter of definition. In the case of starch, the phenomenon is probably not exactly the same as in the case of the efflorescence of an inorganic salt in which the water is held in stoichiometric proportions. Nor does the phenomenon seem to be exactly the same as that which occurs in the case of certain protein crystals which show no x-ray diagram unless great care is taken to prevent efflorescence (111). In many soluble proteins, this process does not seem to be reversible except through recrystallization; in starch it is. One thing seems certain: water is held in starch very firmly. FISCHER (27) found that starch granules treated with a dilute solution of cobaltous chloride ceased to be colored rose red long before they became anhydrous. On the contrary, they turned in-

tense blue, showing that the water of partially dried starch is held so firmly it is not available to the cobaltous chloride.

It is also difficult to understand why the birefringence of the starch granules, as made manifest by the black cross, should persist in anhydrous starch, as we shall see it does, if the crystals weather. The black cross appears because the crystallites are approximately parallel, an arrangement one would hardly expect to persist after weathering. It is therefore doubtful that the behavior of starch when sharply dried is due to exactly the same phenomenon as the weathering of a crystal losing water of crystallization.

Furthermore, it is difficult to explain as a case of efflorescence (95) the loss of the x-ray diagram when natural starch is thoroughly dry-ground in a pebble mill. Our hypothesis, however, offers a basis for a common explanation for both phenomena. This hypothesis assumes that, if there are no effective contravailing forces, starch molecules tend to contract along their long axes, to crumple up. The evidence at hand and already presented does not prove this innate tendency, but it does make our assumption at least reasonable. In the natural moisture-containing granule, the macromolecules are not fully crumpled, because with the adsorbed moisture they occupy too much space for them to contract fully. They are more or less stretched. As the granule is dried, the chains become thinner, occupy less space and in consequence are able to contract further. In the process, they lose their orderly arrangement sufficiently to sacrifice their x-ray diagram. Perhaps the disorderly arrangement arises because contraction is not uniform from macromolecule to macromolecule, and therefore the diffracting groups of individual molecules change their positions with reference to one another from macromolecule to macromolecule. One need not imagine a more extensive disorganization. This idea comes to pretty much the same thing as the second of KATZ's alternatives. In the moist natural granule, the macromolecules are prevented from crumpling because they can shorten only by thickening, and being associated with water, there is no space for them to shorten. If, however, the radial, parallel structure is sufficiently broken up by mechanical disintegration of the granule, there is no longer the impediment to contraction that exists in the intact granule, even in the presence of some moisture. A large number of the macromolecules are then free and the x-ray diagram is more or less lost.<sup>21</sup>

Our hypothesis is also consistent with the changes in x-ray diagram that occur in starch when it is made to swell. It is well known that stretching such fibers as wool or hair changes their x-ray pattern—presumably because

<sup>21</sup> It would be interesting to determine whether dry grinding has an analogous effect upon the x-ray diagram of gelatin. It is possible, for ALSBERG and GRIFFING (5) found it to have a marked effect upon solubility and gel formation. It would also be interesting to examine gelatin films made by drying sols and gels. FIELD (25) has shown that the former are not birefringent as are the latter.

the chain molecules of which they are composed are straightened and lengthened (9).<sup>22</sup> KATZ and co-workers (34, 45-53) have found that starch, when made to swell in water by heating, may undergo alteration of its x-ray diagram, and that in certain cases this alteration is reversible. They suggest that the change is due to the formation of different modifications of starch. The phenomenon might, however, be explained according to our hypothesis as the consequence of lengthening or shortening of the chains.

The hypothesis suggested would also explain the hysteresis which, as we have seen, hinders dried granules from returning to their original size when moistened. According to this hypothesis, the chain macromolecules shorten or crumple as the granule loses water, else the granule would not shrink. With the removal of water, the macromolecules are brought closer together than they have ever been, for it must be remembered that starch is formed in a *milieu*, the cytoplasm, which has a high water content. Unless starch is artificially dried, it always contains a fair amount of moisture. With the removal of moisture and the moving closer together of the contracted but thickening chain molecules, linkages through secondary valence are perhaps formed between side groups that have never before been united. As already indicated, this might also lead to distortion of the lattice and loss of the x-ray diagram. SPEAKMAN (90, 91) has assumed something of the same sort to occur in wool, heated to higher than ordinary temperature, a treatment which reduces the capacity of the fiber to sorb water hygroscopically. The black cross still remains in the anhydrous granule, for probably the contraction of the long axis of macromolecules, even though it distorts the lattice, does not greatly disarrange the parallel arrangement of the macromolecules or distort the crystallites upon which the birefringence depends.

When a granule in this state is moistened, it is necessary that these newly established linkages be broken up, if it is again to swell to its former state. Obviously, this would take time, and would occur imperfectly. Consequently, the granule would swell slowly and not fully to its original size.

We now see why injury to the granule enables it to swell at the site of the injury. The injury separates some of the macromolecules or micelles so that they are no longer surrounded on all sides by other macromolecules or micelles. They associate themselves to their maximum capacity with water. Again no limiting membrane is necessary to account for the phenomena, which are due merely to the fact that through the site of the injury the orderly packing of the chains has been disarranged. ITALLIE (42) has made an analogous suggestion but he also assumes the existence of a limiting membrane. It is plain why the degree of swelling in cold water depends upon the completeness of the disruption of the granules. Our hypothesis also

<sup>22</sup> This view was criticized by KATZ (9, pp. 207-209), to whom ASTBURY (9, pp. 210-211) replied.

explains the observation of KATZ and HANSON (50) that injured granules change the character of their x-ray diagram at a temperature  $10^{\circ}$  to  $20^{\circ}$  C. lower than that at which uninjured granules undergo this change, and also why injury wipes out to a large extent differences in this temperature which are shown by different species of starch in the uninjured state.

### GROWTH

Structure of the type here suggested is consistent with what is known of the manner of growth of starch grains. If starch grains possessed an outer limiting membrane permeable only to substances of comparatively low molecular weight, it would be difficult to understand how small granules grow into large ones. There is firstly the difficulty of imagining how a limiting membrane of the type demanded by the properties of the natural granule stretches and enlarges simultaneously with the growth of the granule. Certainly, the long molecules of starch, if formed in the protoplasm outside, would find it difficult to pass through such a membrane. It would, therefore, be necessary to assume that mono- or disaccharide passes from the protoplasm across the membrane into the granule there to be united into long chains and oriented radially. This would demand that the directive forces residing in the cytoplasm act across the membrane, or else that cytoplasm itself extends through the membrane into the granule. For such assumptions, there does not as yet seem to be evidence. If, however, there is no limiting membrane of the sort under discussion, but the cytoplasm—perhaps in a condensed form—lies in contact with the growing granule, then it is simpler to picture the growth of a granule much as SPONSLER (92, 93, 94) explains the formation of a cellulose fiber in the cell wall or FARR and ECKERSON (22) explain the formation of the cotton fiber.

A granule might grow through lengthening of the starch chains by the condensation of a glucose or maltose residue at the peripheral end of the chain, so that the chains grow longer. Or perhaps the method of growth is more like that described for cotton fibrils by FARR and ECKERSON. In that event, small starch crystallites are formed in the cytoplasm surrounding the small granule. These then arrange themselves radially around the granule and become attached to or inserted between the chains of particles already arranged radially in the growing granule.

As the granule grows, the chains must lie less closely packed at their peripheral ends. This leaves room between them for the formation of new chains by the same process by which the original chains were formed; and these new chains are also radially oriented between the outer ends of the older chains. Thus, crystallites are built up which are irregularly conical with their bases lying at the periphery of the granule. Such a form is clearly to be seen

in unlaminated starch in which the crystallites extend from the periphery to the hilum (12), a phenomenon which can be explained only on the assumption that new starch substance is added at the base of the needles.

This picture of the growth of starch granules is not the same as that suggested by ZWIKKER (112), but it is not inconsistent with it. ZWIKKER suggests that the leucoplast secretes a concentrated colloidal solution of amylose and amylopectin. Now starch lowers the surface tension of water, so that during an interruption of the activity of the leucoplast there is at first a concentration of the substance with the greatest effect on surface tension (*e.g.*, a potassium salt of amylopectin) at the surface accompanied by its separation or precipitation. When the leucoplast again becomes active, this layer remains unaffected and the process is repeated. ZWIKKER, who wrote in 1921 when the x-ray analysis of organized structures had not been developed as it is today, has nothing to say regarding the formation of colloidal amylose or amylopectin. Now that we know something regarding the form of the starch unit, we can go a step farther and extend the picture to the manner of formation of the chains. It is entirely possible that surface forces play a rôle, as ZWIKKER suggests, in determining the parallel arrangement of the chains in building up particles of the type observed in the case of cellulose fibers by FARR and ECKERSON.

If this is in fact the mechanism by which starch granules grow, there is no reason to believe that the chains or macromolecules of which the crystallites are composed are all of the same length. Indeed, the chances are greatly against it. It is probable that starch, like many other materials (*cf.* 98, pp. 109, 112–113; also 65), is composed of macromolecules of varying length. NEALE (72) has presented evidence that this is true for the cellulose fiber. There is much evidence that this is in fact the case for starch. Thus FOUARD (29; *cf.* 79, 81) has reported that solutions of demineralized starch can be fractionated by dialysis through collodion membranes of different permeability. The occurrence of chains of different lengths is also consistent with the views of HANSON and KATZ regarding the structure of the granule. Probably the arrangement of the molecules in starch granules resulting from the manner of growth above suggested causes imperfect packing and may be a factor leading to the loose structure of granules already discussed.

Whether the elements in the unlaminated starch of BAKHUYZEN extend, as they appear to do under the microscope, from the hilum to the periphery, it is not possible to decide from existing evidence. Some of the starch elements may be as long as this and consist of macromolecules which are joined together by van-der-Waal forces to form quite long fibers. This is well known to occur in the case of soaps, and has been suggested for starch (10). Moreover, it should be noted that SPONSLER believes that the diffraction data obtained from x-ray analysis of cellulose may be accounted for upon the basis of chains of cellulose-unit cells of indefinite length.

## THE LAMINATED STARCH GRANULE

BAKHUYZEN correlates the occurrence of the large needles in starch from wheat plants grown under constant conditions with the absence of lamination or rings. In field-grown wheat seed he finds the needles or trichites no larger than 5 microns, or about 10 per cent. of the diameter of the wheat-starch grain. Ten to 20 or more rings can be counted. Larger needles may also be observed. If such starch is carefully warmed to swelling temperature, the laminae may be observed to consist of small particles. Particles in successive rings may often be seen to be oriented along a straight radial axis, suggesting that such a string of particles was formed from a single needle which had been fragmented by heating. The parts of the needles which correspond to the non-refractive rings or laminae, however, remain.

Much evidence indicates that laminated starch is built up of bundles of parallel chains combined to form crystallites of a length about equal to the width of the laminae. If starch granules develop in the manner described for cellulose fibers by FARR and ECKERSON (22), as we have suggested, then it is quite probable that in some starches the thickness of laminae is determined by the length of the starch crystallite formed in the cytoplasm. Certainly in some, the breadth of the laminae is of the same order of magnitude (1 to 2 microns) as the longest diameter of the cellulose particle of FARR and ECKERSON (*cf.* also 23). In eccentric starches, the laminae at the pole nearer the hilum may be very narrow, indeed. If the growth of the starch granule takes the course suggested by FARR and ECKERSON for cellulose, then any periodicity which interrupted the process must of necessity lead to the deposition of starch substance in layers or laminae.

This view, based on the observations of BAKHUYZEN and others, is in harmony with the observations of HANSON and KATZ (34) who, as we have seen, describe the granule as consisting of short elements radially arranged and embedded in an amorphous matrix.

## SOLUBILITY

It has been known since the first part of the nineteenth century that, while starch granules do not dissolve in cold water, a starch granule which has been injured by cracking, chipping, or grinding disperses more or less<sup>23</sup> in water without warming. It has furthermore been shown that the degree to which the granule disperses depends upon the extent to which it has been disintegrated mechanically, for with very thorough grinding all the  $\beta$ -amylase can be dispersed and extracted from corn starch by cold water (85, 102). Those who, like MATTHEWS and LOT (61), believe that only a minor part of the substance of injured granules is soluble in cold water are cer-

<sup>23</sup> Literature to 1895 in MEYER (64, p. 18); to 1910 in REICHERT (76). Literature not cited in the above volumes (3, 5, 16, 21, 28, 35, 36, 41, 54, 95, 102, 108, 112).

tainly mistaken. This solubility in cold water which results from injury to the granule is not due to alteration of the starch substance by frictional heat engendered in disrupting the granule (41, 43). Nor is it at all likely, as SAMEC (80) states, that grinding as such breaks up the starch complex into smaller aggregates, which disperse readily; the manipulation necessary to injure a granule so that some of its substance disperses is far too slight. All that is necessary is to put a little dry starch on a microscope slide, cover it with a glass cover slip, and press gently upon it for a moment, at the same time moving the cover slip a trifle sideways.<sup>24</sup> Moreover, as we have seen (p. 309), merely drying sharply causes radial rifts which render some of the granule soluble.

Furthermore the colloidal solutions obtained from potato starch ground in a pebble mill do not behave as though the starch had been broken up into smaller complexes. The solutions are strongly opalescent and change rapidly. The concentrated solutions first obtained separate a solid phase until an equilibrium is apparently established such that less than 1 per cent. of starch remains in a non-opalescent or but slightly opalescent solution (24, 25). The phenomenon bears a close resemblance to that exhibited by gelatin and studied by STRAUP (100). For different starches, the equilibrium seems to be a little different with respect to the concentration of dissolved starch at the final equilibrium and to depend to some extent upon the total mass of starch in the system. Some starches require a longer time to reach equilibrium than others, and the longer the time the more difficult it is to obtain non-opalescent solutions. These, for example, are more easily obtained from maize and wheat starches than from potato starch (4, 6, 26).

Examination with the microscope shows that the dispersal of starch from an injured granule is preceded by localized swelling at the site of the injury; it does not occur anywhere else. The dispersing starch does not stream out

<sup>24</sup> On the other hand, it must be admitted that severe dry grinding affects the properties of gels. Gelatin is but slightly soluble in cold water. If some gelatin such as is used by bacteriologists be ground dry for a long time (50 hours) in a porcelain pebble mill, it yields an opalescent solution when suspended in cold water. This solution filters with difficulty but the small quantity of filtrate is perfectly clear and not very viscous. On standing overnight, it sets to a clear gel (*cf.* 5). It may perhaps also prove significant that SCHOCH (85) prepared dispersions from potato starch ground for 500 hours, which did not readily retrograde, and found that 900 hours of grinding produced a certain amount of dextrinization, as evidenced by reddish iodine coloration. Moreover, TAYLOR and SALZMANN (104) found that long grinding in the presence of moisture affected and modified the stability of starch or amylose toward alkali. Possibly such severe treatment is accompanied by a change of some of the starch from the crystalline to the vitreous state as these states are defined by BEILBY (15). This change is sometimes accompanied by changes in solubility and in heat of solution. Grinding a crystal or hammering a metal tends to cause some parts of it to flow. If a substance cannot flow by local melting, perhaps it undergoes some localized form of decomposition. Starch does not melt.

into the surrounding water as though out of a hole in a bag. Rather it behaves as though at the points of injury it were swelling by imbibition. When the imbibition has reached a certain stage, dispersion gradually occurs. The swollen portion never wholly disperses, even after days; nor does the swelling extend into the interior of the granule. The reason why cold-water solubility is related to the degree of disruption of the granules is now clear. Unless the granule has been very completely disrupted, only those portions swell that have been disintegrated; and swelling seems to be a necessary step antecedent to dispersion. It is obvious that the localized dispersion of injured granules is as difficult to explain by the assumption of a membrane or related restraining mechanism as localized swelling—and for the same reasons. It would seem that our hypothesis regarding the structure of granules, if it is satisfactory to explain the swelling phenomena, is equally satisfactory to explain why an intact granule does not disperse, but disperses at once at the site of injury when it is mechanically damaged. Injury having more or less disarranged the close-packed radial parallel chains, some of these are free to associate themselves with water along their long axes and ultimately to disperse. In this connection it may be noted that NÄGELI (70, pp. 111–112) reported that saliva attacks granules tangentially, *i.e.*, transversely to the radius of the granule, more readily than in the direction of the radius. This is what one would expect if in fact the starch chains lie radially.

#### OPTICAL PROPERTIES

Some investigators have assumed that the material in a moist natural granule, being swollen, is under a strain. To this strain, they attribute the appearance of the black cross which is so characteristic when a starch granule is viewed through the polarizing microscope with nicols crossed (20). The alleged disappearance of this cross when the granule is dried has been regarded as proof of this view. As HARRISON (36) puts it: "Since the granules have a resistant outer coating, any swelling of the interior portions will produce an increase in the internal pressure. When the outer coating is broken, and this occurs when the granules are ground in a mortar, no pressure will be produced by the swelling of the interior portions, but under these conditions no cross is shown by the granules."

HARRISON's observation that dry starch granules swell when moistened is undoubtedly correct, as we have seen, and so is his observation that disrupted granules may not show the black cross in polarized light. But the assumption which he and many others make that "the granules have a resistant outer coating" has been shown above to be unwarranted. His statement that the black cross becomes indistinct, if the granules are dried, is also unwarranted. PURIEWITSCH (74) has reported that arrowroot starch dried for 76 hours at 105° C. has exactly the same appearance in polarized light as wet



starch immersed in water for 2 days. KATZ and DERKSEN (48, p. 109) made a similar observation.

It is probable that HARRISON was deceived, because it is difficult to get a clear picture under the polarizing microscope of starch examined in air. Therefore, GRIFFING (31), at the writer's suggestion, heated potato starch for several days to a little over 110° C. It was then transferred as rapidly as possible, and still hot, to anhydrous glycerol or to cedar oil and examined immediately in these media; it exhibited perfectly normal birefringence.<sup>25</sup>

It has long been known that when starch is gelatinized by heating in water its birefringence is lost and the black cross disappears. Indeed, the loss of birefringence has been proposed as a criterion for fixing the gelatinization temperature (76, p. 298); but WOODRUFF and WEBBER (110) found that the temperature at which birefringence disappears varies with the amount of water available for swelling.

As already pointed out, HARRISON (36) has shown that the black cross disappears when starch granules swell in the cold as the result of injury. HUSS (41) made similar observations and showed that the black cross vanishes whenever the starch granule is made to swell beyond its normal size, whatever the cause. He pointed out that the disappearance of the black cross and the taking up of dyes are parallel phenomena.

Indeed, mechanical disruption greatly modifies the optical properties of granules. HARRISON's observation that grinding starch in a mortar causes the black cross to disappear has already been mentioned, but the observation is much older (67). Indeed, SCHEFFER (82) has proposed examination in polarized light as a method of detecting injury to starch granules because such injury is difficult to recognize in ordinary light. In polarized light, injured granules appear either with but slight birefringence or else with only a part of the granule birefringent. The fact that birefringence may be lost only in part of the granule and the black cross remain in part is probably the reason why a few investigators<sup>26</sup> have reported that injury does not obliterate the black cross. The truth seems to be that just as the degree to which a granule is soluble in cold water and swells in it depends upon the completeness of the disruption of the granule, so also does the loss of birefringence. Badly shattered granules lose their black cross and most of their birefringence, but examined with a selenite plate some birefringence may still be detected (31), and starch that has been severely ground loses its x-ray diagram (66, 95).

The localized loss of birefringence in injured granules may be demonstrated by water extraction of starch ground for many hours in a pebble mill

<sup>25</sup> FISCHER (27, p. 83), however, states that dried granules do not show lamination but appear homogeneous, and so does NÄGELI.

<sup>26</sup> For example, NÄGELI (68).

until it appears to have lost the black cross. The ground starch is suspended in water and separated from the solution by centrifuging. The process is repeated till the extract is not blued by iodine. The undissolved residue, dried with alcohol and ether and examined in polarized light, appears dull white on the dark field, looking much like the ground starch before it was washed, except that there had been washed away the amorphous, granular, non-luminous material which had been adherent to the surface of the granules. When this material was mounted in cedar oil, the granules appeared dull yellow, transparent, with numerous traces of bright birefringence and distorted black-cross markings. The explanation is obvious. Grinding covered the surface of most granules with abraded starch substance, which is not birefringent or only slightly so, and therefore masked the birefringence of the material in the interior of the granules. After this had been washed away by extraction with water, the granules again appeared birefringent, although this was less pronounced and no longer typical because the granules for the most part were more or less affected by the treatment.

Apparently, anything that sufficiently breaks up the anatomical structure of the granule greatly modifies its birefringence, if it does not wholly destroy it. It is, therefore, probable that at least some of the optical properties are due to parallel arrangement of crystals or other elements in the granule. This is also the opinion of KATZ (47).

We must, then, distinguish two sorts of birefringence in the starch granule—that which is due to parallel arrangement of the starch crystals, and that which is inherent in the starch substance itself and still visible with a selenite plate in badly shattered starch. The latter is relatively slight.

The black cross is due to the parallel arrangement of the starch crystallites manifest through the microscope in the radial striations. It is due, therefore, to a comparatively coarse structure and disappears whenever this is disorganized. Hence, it is understandable why the black cross remains in completely anhydrous starch, which, according to KATZ, no longer exhibits an x-ray diagram. In such a dry granule, the radial striation and the laminations are still visible. Conversely, when a starch granule is gelatinized without enough water to permit free swelling,<sup>27</sup> the radial striations, the laminae, and the black cross are no longer visible but the granules still exhibit an x-ray diagram, although an altered one. It is obvious, then, that the disorganization which causes loss of the capacity to exhibit an x-ray diagram is of a finer sort than that which is sufficient to destroy the black cross.

The hypothesis herein advanced is, of course, to a considerable degree a speculation, but a speculation which seems consistent with and capable of offering a reasonable explanation of all the facts known to the writer. It

<sup>27</sup> KATZ terms this "first-degree gelatinization."

emphasizes the organization of the granule without consideration of which it seems impossible to form any sort of rational mental picture of the behavior of the starch granule. The importance of the organization of biological structure in determining behavior has been emphasized by others (40). The hypothesis offered may in the end prove to be ill-founded, but it poses many questions which can be answered by experiment and this is sufficient justification for its presentation.

FOOD RESEARCH INSTITUTE

STANFORD UNIVERSITY, CALIFORNIA

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# A CHEMICAL AND PHYSIOLOGICAL STUDY OF TRAUMATIN, A PLANT WOUND HORMONE

JAMES BONNER AND JAMES ENGLISH, JR.

(WITH THREE FIGURES)

## Introduction

The first tentative formulation of the "wound substance" concept was made by WIESNER some 50 years ago. In his "*Elementarstruktur*" (25) he suggested that it might well be that *substances* formed or produced by wounded cells pass from these cells to neighboring uninjured tissue and there bring about such phenomena as callus formation and regeneration; bring about, in short, a resumption of meristematic activity by cells apparently mature. Sound experimental evidence in support of the wound substance hypothesis was obtained by HABERLANDT (5-9). His classical potato experiment should be particularly stressed. He found that discs cut from a potato tuber showed cell divisions leading to periderm formation only if (a) phloem and (b) "wound hormone" were present. This wound hormone appeared to come from contents of injured cells at the cut surface of the disc. In a number of different ways HABERLANDT demonstrated that both the influence coming from the phloem and that coming from the surface are diffusible chemical substances and that the interaction of the two is necessary for renewed division of the mature parenchymatous cells of the potato tuber, as well as of the kohlrabi root (8). In a later publication (8) HABERLANDT showed that in other cases also, division of mature cells may be induced by the cooperation of "lepto-hormone" and "wound hormone." By judicious dissection of the leaves of succulents, surfaces of uninjured cells could be exposed. The cells of these uninjured surfaces were capable of responding to the application of tissue juice from other leaves with a vigorous renewal of cell division activity.

REICHE (19) confirmed the results of HABERLANDT in another way. She injected the petioles and stems of various plants (Nymphaeaceae, *Solanum*, *Gratiola*) with dilute tissue extracts and found that cell divisions were induced wherever the *uninjured* cells of the stem or petiole came in contact with the extract of *injured* cells. She was inclined to the view, however, that the activity of the extracts resided in suspended cell fragments rather than in soluble substances. WEHNELT (23), whose work with the parenchymatous lining of bean pods will be discussed more extensively later, found that these intact parenchymatous cells react rather to a water soluble, heat stable substance present in tissue extract. Another favorable object for the demonstration of wound hormone activity was found by WILHELM (26) in the parenchymatous lining of the hollow stem of *Vicia faba*.

It may be concluded that it is a well established fact that when a plant is injured, substances are formed or liberated which are capable of causing other uninjured cells to resume active growth. These substances may well be called wound hormones since they are carriers of correlation between one portion of the plant and other portions. They appear in some cases to be also "cell division substances." It is equally well established that in many cases the wound hormone acts only in conjunction with a second factor contained in the phloem. This factor, however, is less well known and is less amenable to study than is the wound hormone itself.

The chemical nature of the wound hormone has not been studied previously in any detail. In fact, its very existence as a chemical individual might be considered as thrown into doubt by the work of WEHNELT and WILHELM, who have shown that the most diverse and ill assorted substances possess some typical wound hormone activity. The chemical properties of the active principle of tissue extract are also in question since it has been reported by HABERLANDT that it is heat labile, by WEHNELT that it is heat stable, and by REICHE that it is not soluble in water but is heat stable. In this connection it should be remembered that different test objects may actually respond to different wound hormones.

That the wound hormone possesses considerable interest both from the theoretical and the practical points of view is perhaps obvious. In the wound hormone we have a substance which is capable, under suitable circumstances, of bringing about renewed growth activity of otherwise mature cells. The rôle of wound hormone in wound healing, parthenogenesis, adventive embryony, callus formation, etc., has been discussed in detail by HABERLANDT (9) and need not be gone into here. Attention has been called more recently to the rôle of wound hormone in the culture *in vitro* of plant tissues (2). In the present work, an attempt has been made to work out *one quantitative* test for wound substance activity, and, using this test, to purify the active principle. The work, therefore, has been confined to one test object. The test was developed from that suggested by WEHNELT (23). Since the work was undertaken in the fall of 1935, several papers bearing on the subject have appeared (13, 15, 17, 20, 22). These will be discussed later as the appropriate connections arise.

## Experimentation

### PRINCIPLE OF THE TEST

The use of the immature bean pod for the demonstration of the action of wound hormone is due to WEHNELT (23), who worked out a qualitative activity test and determined a number of facts concerning the physiology and chemical properties of the active principle.

The immature bean pod merely need be slit lengthwise along suture and

midrib, and the unripe seeds removed, to expose the layer of uninjured parenchymatous tissue which lines each seed chamber. If this layer of tissue is then injured, for example, by a prick with a needle, the cells in the region of the wound divide and enlarge so that a small "neoplasm" or intumescence projecting above the level of the surrounding tissue is formed. A much more striking result may be obtained by the application of juice from crushed beans. If a drop of such bean juice is applied to the uninjured surface it is absorbed in about 24 hours. Under the point of application of the drop and before it is completely absorbed, a cylindrical intumescence begins to arise, which may attain a height of as much as 3 mm. in the course of 48 hours. Histologically the intumescence consists of cylindrical parenchymatous cells elongated at right angles to the surface of the seed chamber.

The change in wall structure of these cells during the course of the reaction is a point of some interest. The normal parenchyma of the seed chamber consists of approximately isodiametric cells and these possess the foliar arrangement of cell wall micellar units which is typical of such cells (1). They are isotropic when viewed in tangential section between crossed nicols of the polarizing microscope. During the course of the wound hormone reaction the shape of these cells changes to cylindrical due to the extensive cell elongation which takes place. The elongating cells are anisodiametric and they show the corresponding anisotropy of cell wall structure which is typical of elongating cells. The micellar units are arranged in a direction which is, statistically, perpendicular to the axis of elongation (1).

Normal mitotic cell divisions occur in the new growth, their frequency depending upon the species and variety of bean used. In a given variety of bean the frequency of cell division depends upon the concentration of the applied extract as does the height of the intumescence. With increasing size, the total number of cell divisions increases. The intumescence is thus a *product* of simultaneous cell division and cell enlargement. In our experience those varieties of beans which give large intumescences in response to the addition of wound hormone also show extensive cell division, whereas those varieties which give intumescences of limited size may show few or no cell divisions. As far as has been observed during the present work, cell divisions appear to be essential to the formation of large amounts of new growth. Previous authors who have investigated the wound hormone response of the bean pod have interested themselves chiefly in this cell division activity (23, 13). The quantitative estimation of the latter, however, is at best tedious and hence difficult of application to large scale routine testing. Furthermore, investigators who have *confined* themselves to estimation of division frequency, as will be shown later, have been misled on important points. In the present investigation the *height* (size) of the intumescence, a readily and quantitatively determinable quantity, has been chosen as the criterion of wound substance activity.

## THE QUANTITATIVE TEST

It was found quite possible, with the observance of a number of precautions, to place the "bean test" upon a quantitative basis. A large and constant supply of beans was essential. This was obtained through the cooperation of the Lake Farm Produce Co.<sup>1</sup> The beans (Kentucky Wonder) to be used on any given day were selected from a fresh, high grade stock (one pound chosen from 50 to 150 pounds). Only uniformly firm, immature (not rounded out by the developing seeds) pods of dark-green color were selected. On those days when fresh beans were not available, stock which had been stored at 35° F. was used. Such pods, however, gave less reliable results, apparently owing to the fact that they were extensively dried out. Tests were made at the same hour each day in order to avoid, among other things, effects of diurnal fluctuation such as are found in the *Avena* test (14).

The pods were slit lengthwise along suture and midrib (fig. 1), opened, and the seeds removed. The individual seed chambers were then cut from the pod and arranged in Petri dishes upon moistened absorbent paper. The apical chamber and the basal one from each pod were rejected since these are less reactive than the others. The "cups" were arranged in five columns of six cups each. Each column consisted of chambers from a single bean; each row, of chambers from different beans. In each Petri dish there were, therefore, cups from five different beans, six cups from each bean, and in each dish it was possible to test six different solutions on five different beans. Because five beans did not suffice in general to give an accurate measure of activity, due to individual variations, ten beans were used in most determinations. In tests requiring special accuracy (as, for example, molecular weight determinations by the diffusion method (4, 24, 12) twenty or more beans were used. The solutions to be tested were diluted with distilled water instead of nutrient solution, since it was found that the use of a balanced salt solution made no difference in the results. Drops of the test solution (approx. 0.01 cc. per drop) were placed in the center of each cup with a micropipette. The tests were then placed in an incubator at 25° C. At the end of the test period (in general 48 hours, see below), each cup was sectioned through the reacting portion and the height of intumescence measured under a low power binocular microscope.

## COURSE OF THE REACTION WITH TIME

Figure 2 gives the course of the reaction with time for a series of dilutions of the same stock solution. The first reaction appears between 8 and 16 hours, before the drops have been completely absorbed. Each concentration

<sup>1</sup> The authors are deeply indebted to Mr. K. WADA of the Lake Farm Produce Co. Only through his constant cooperation and assistance was it possible to obtain the supply of fresh high quality beans needed for the routine testing.

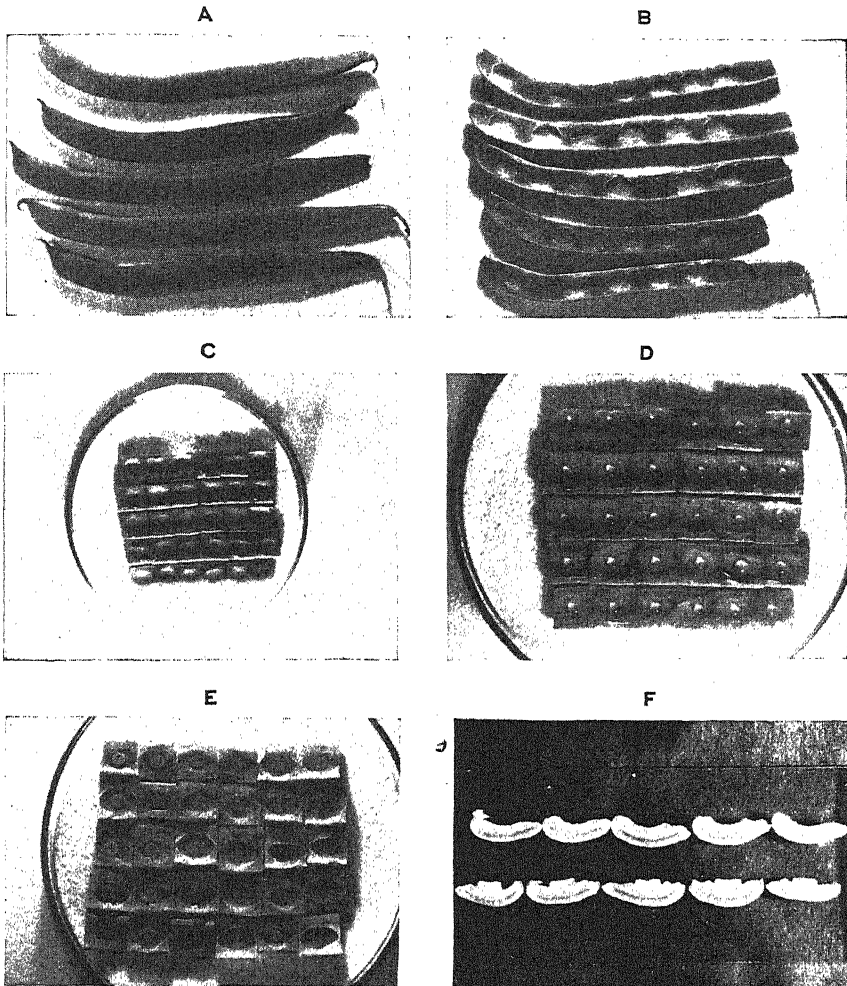


FIG. 1. Steps in the execution of the "bean test": A, fresh beans; B, beans opened and seeds removed; C, individual seed chambers arranged in Petri dish; D, drops (containing traumatin) in place; E, characteristic reaction to traumatin, after 48 hours; F, cross section through the seed chamber after 48 hour test. The top row is a typical control, and the bottom row a reaction which is shown in the linear portion of fig. 3.

gives a typical S-shaped growth curve, the curves from tests with smaller concentrations having lesser slopes and earlier maxima than those from tests with the higher concentrations. The highest concentration, for example, attained its maximum only after 64 hours while the lowest concentration attained its maximum after 40 hours. Since, for routine testing, it was desirable to have as short a test period as possible, 48 hours was chosen as

the standard length of incubation. Figure 3 gives a typical example of concentration plotted against height of intumescence after 48 hours. It may be seen that:

1. Height is proportional to concentration between approximately 0.20 and 0.67 mm. Over this range, then, height of intumescence, less the correction due to the fact that the curve does not pass through the origin, may be used as a measure of wound hormone activity. If a longer time is chosen, the linear relationship holds over a wider range.

2. The curve does not pass through the origin. If the main linear portion is extrapolated, it passes through the height axis at about 0.18 mm.

This "intersection point" (I, on fig. 3) is of fundamental importance to

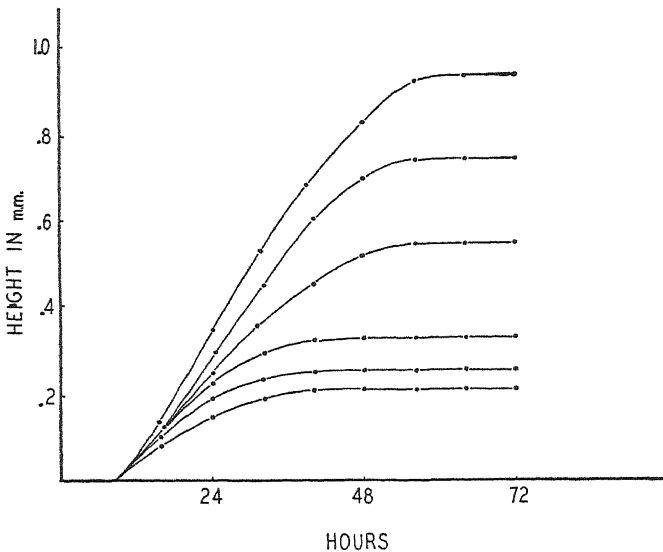


FIG. 2. Development of intumescence with time. Six concentrations of traumatin, each one-half as great as the preceding, are represented.

the understanding of the test. The height of intumescence corresponding to the intersection point varies from day to day, but on any given day it is equal to the maximum response which is given to any of the "non-specific" agents which are discussed below. It is to be noted, however, that non-specific agents may cause a response *smaller* than this; thus water causes a response which is several times smaller than the *maximum* non-specific response which is possible. Above this non-specific portion of the response is superimposed the major effect of the added hormone. Only those substances which in some concentration elicit a response greater than the non-specific portion, can be considered as possessing true wound hormone activity. The failure in the past to distinguish sharply between specific and non-spe-



cific response may have been due to the much smaller accuracy of the cell division frequency determinations which have been made. In any case it has led to the present confusion as to the specificity of the hormone, a question which will be discussed later.

The wound hormone itself is capable of causing the non-specific as well as the specific response. That this is so, is clear from the activity-concentration curve (fig. 3) in which one is superimposed on the other. The non-specific

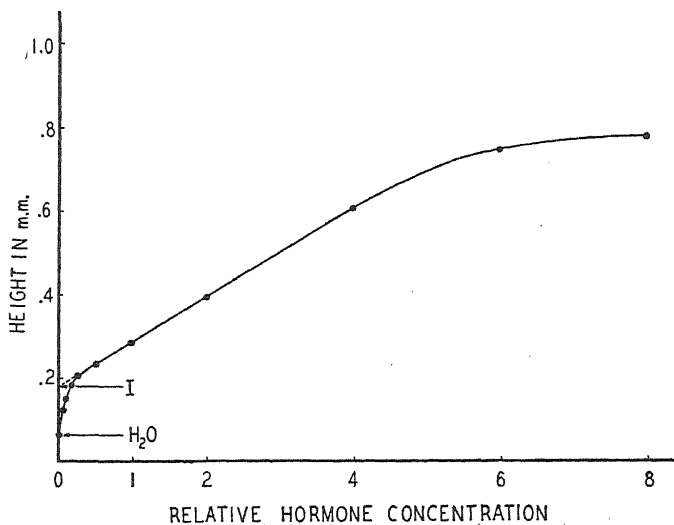


FIG. 3. The relation between traumatin concentration and height of intumescence. I, "intersection point" (see text); H<sub>2</sub>O, the reaction to pure water.

portion is brought about by much lower concentrations of wound hormone than is the specific response, and also it is caused by much smaller concentrations of wound hormone than of the various non-specific agents. This fact suggests a second measure for wound hormone activity, namely, the lowest concentration which is just capable of causing full non-specific response. This measure of activity does *not* give relative activities for two wound hormone preparations identical with those obtained by the first method (from large intumescences), as will be shown later.

#### THE NON-SPECIFIC REACTION

It has already been mentioned, that WEHNELT (23) found a number of agents other than the juice of beans, capable of causing a reaction in the bean test. WEHNELT was often troubled with a considerable reaction caused by the application of drops of plain water. He also found that sugars, neutral salts, and even agar regularly caused reaction. It is desirable to make clear at once the relationship between these non-specific reactions

and those due to the application of wound hormone. Water alone gives a reaction particularly if the test tissue is relatively dry. The standard "fresh" beans<sup>2</sup> which have been used in this investigation give, in general, either no response or a negligible one to the application of pure water (0.07 mm. is a value obtained on many days). If such beans are allowed to dry out, however, they react more vigorously to water. Sugars and neutral salts give, with these beans, reactions greater than that of water only when used in high concentrations. Thus 10 per cent. glucose gave a reaction four times as great as that of water, while 1 per cent. gave only the reaction of water alone. The more permeable substance, urea, in equivalent concentrations gave reactions only half as great as those due to glucose. The effects of these substances are clearly osmotic ones. Another class of non-specific responses includes those due to wounds or to the application of markedly toxic substances. In either of these cases there is a circular zone of response around the dead or badly injured portion, and a crater-shaped intumescence is formed.

It is clear that the non-specific causative agents may be classified under two general headings: (1) Those causing slight if any visible injury such as hypo- and hyper-tonic solutions; (2) those causing severe injury, either mechanical (pricks, etc.) or chemical ( $\text{HgCl}_2$ , strong acid or alkali, etc.). In these cases it is clear from the crater shape of the intumescence that the injured cells have liberated wound hormone which caused adjacent cells to react.

The causative agents which superficially appear to be non-specific are probably all based upon the liberation of wound hormone within the tissue itself. The maximum amount thus liberated by agents which are not visibly toxic is then just sufficient to cause a maximum intersection point non-specific response. Intumescences caused in this way are not to be confused with those caused by the addition of wound hormone from outside. The average maximum response due to the former is 0.10 to 0.30 mm., that due to the addition of wound hormone, 1.9 mm. or more.

#### REACTIVITY OF DIFFERENT VARIETIES OF BEANS

A number of leguminous pods other than those of *Phaseolus* were tested as to their suitability for the bean test. Pods such as those of pea, lima bean, and chinese pea, which have only rudimentary parenchymatous linings of the seed chambers give no reaction whatsoever. This is not surprising since the reaction is carried out by the parenchymatous lining of the bean. Different varieties of the string bean itself also differ in their response to wound hormone. The maximum average height of intumescence for five varieties

<sup>2</sup> By this is meant fresh market beans. Beans fresh from the plant were found to be improved by a slight amount of drying (2).

is recorded in table I. Kentucky Wonder, brown seed, is at least four times more reactive than is Kentucky Wonder, white seed, and excels the other varieties still more. The brown seed Kentucky Wonder was, therefore, used exclusively for the quantitative test outlined above.

TABLE I

MAXIMUM HEIGHT OF INTUMESCENCE GIVEN BY DIFFERENT VARIETIES OF BEANS

VARIETY	MAXIMUM AV. INTUMESCENCE HEIGHT
	<i>mm.</i>
Kentucky Wonder, brown seed .....	1.9
Kentucky Wonder, white seed .....	0.45
Florida Black Valentine .....	0.3
Golden Wax .....	0.15
Green Pod .....	0.00

Some investigation of the reasons underlying this varietal difference in reactivity has been made. In table II are given the results of reciprocal tests between Kentucky Wonder brown seed, and Florida Black Valentine. The

TABLE II

RECIPROCAL TESTS BETWEEN KENTUCKY WONDER AND FLORIDA BLACK VALENTINE BEANS

EXTRACT USED	HEIGHT OF INTUMESCENCE	
	KENTUCKY WONDER BEANS	BLACK VALENTINE BEANS
	<i>mm.</i>	<i>mm.</i>
Kentucky Wonder .....	0.65	0.30
Black Valentine .....	0.51	0.30
Kentucky Wonder + Black Valentine .....	0.66	0.30
Free Acid Conc. ....	0.70	0.0

latter contains wound hormone which is highly active on Kentucky Wonder but only slightly active on itself. Kentucky Wonder juice also has only slight activity on Valentine. A mixture of the two juices shows that Valentine juice contains no inhibitor of the reaction. The fraction of the juice which is active on Kentucky Wonder and which was purified during the course of the present work possesses little, if any, activity on Valentine. A possible explanation is that the interaction of several factors is necessary for the response and that we have purified only one of them. The others, apparently, are present in the Kentucky Wonder bean in considerable amount, but are lacking in the Valentine. A complete elucidation of the reasons for the difference must however await a further investigation.

## DISTRIBUTION OF THE WOUND HORMONE

That the wound hormone is widely found in nature has been indicated by the work of WEHNELT (23) as well as that of others. A more extensive investigation of its distribution has been made during the course of the present investigation and a portion of the results are presented in table III.

TABLE III

OCCURRENCE OF THE WOUND HORMONE

SOURCE	ACTIVITY	SOURCE	ACTIVITY
Bean pod .....	++	Corn meal .....	-
Brussels sprouts .....	++	Soy bean meal .....	-
Sweet potato .....	+	Wheat germ .....	+
Potato .....	+	Molasses .....	+
Orange .....	++	Yeast, bakers' .....	-
Lemon .....	++	Yeast, brewers' .....	+
Tomato .....	++	Vitamin B concentrate .....	-
Lettuce .....	++	Urine, human .....	-
Spinach .....	+	Urine, cow .....	-
Pea plant, etiolated .....	++	Peptone, Difco .....	-
Pea plant, green .....	++	Beef extract .....	-
Pea seed .....	-	Liver extract .....	-
Hay, alfalfa .....	+	Milk .....	-
Malt .....	+	Egg albumin .....	-
Rice polishings .....	-	Serum .....	-
Cabbage .....	+	Emulsin .....	-

Each source was tested over a wide range of concentrations. A substance listed as inactive failed to cause any response other than non-specific in any concentration. Of the animal tissue and extracts examined, none was found to possess any marked activity. The reaction to serum obtained by WEHNELT was, presumably, merely non-specific in nature. The urine of cows is of particular interest. Alfalfa hay is a relatively rich source of the hormone (table III). The urine from cows fed this hay, however, is completely inactive. Wound hormone behaves, in this respect, quite differently from auxin a, which is ingested by the animal and excreted in the urine. The "sporogenes vitamin" (18) also is found in the urine of herbivores. Wound hormone, however, either is destroyed by the flora of the gut, or is metabolized further by the cow.

The richest sources were found to be green beans themselves, leaves in general, dry brewers' yeast, and the juice of oranges and tomatoes. Attention might be called to the fact that various samples of brewers' yeast were all inactive, in marked contrast to dry brewers' yeast. Although green portions of the plant are rich in wound hormone, the latter is not confined to

chlorophyll-containing parts. Thus, it is to be found in fruits, in potatoes, and in wheat germ. It is not present in any considerable amount in the pea seed, but is formed after germination and during growth of the seedling. Seedlings grown in darkness contained as much wound hormone per unit dry weight as did those grown in the light.

Table IV gives a quantitative comparison of a few of the more promising

TABLE IV  
A QUANTITATIVE COMPARISON OF SOURCES RICH IN WOUND HORMONE

METHOD OF EXTRACTION	SOURCE	RELATIVE ACTIVITY PER MG. DRY WT. OF EXTRACT
Water extraction	{ Bean pod .....	100
	{ Pea plant .....	77
	{ Orange .....	50
	{ Yeast, brewers' .....	31
	{ Alfalfa hay .....	15
Absolute alcohol extraction	{ Bean pod .....	410
	{ Yeast, brewers' .....	380
	{ Alfalfa hay .....	233
	{ Wheat germ .....	143

sources of the hormone. In no case was a source found which was richer than the bean pod itself. This is in accord with the findings of SILBERSCHMIDT and KRAMER (20), that the wound hormone content of a plant tissue, as measured by the bean test, is greater the closer the relationship of the plant in question to the bean. However, brewers' yeast which is certainly systematically far removed from the bean yields a very active alcoholic extract.

It might be mentioned here that a number of different methods of extraction of the wound hormone have been compared. The results of a typical experiment are presented in table V. The hormone is heat stable and the

TABLE V  
A COMPARISON OF DIFFERENT METHODS OF EXTRACTING WOUND HORMONE FROM BEAN PODS

METHOD OF EXTRACTION	PREPARATION OF MATERIAL	RELATIVE ACTIVITY	YIELD: % DRY MATTER/DRY WT.	TOTAL AMOUNT OF HORMONE
Water extraction	{ Fresh: ground .....	1	41	41
	{ Ground: dried .....	1	44	44
	{ Dried: ground .....	1	48	48
Alcohol extraction	{ Ground: dried .....	4.1	} 4	16.4
	{ Dried: ground .....	4.1		

fresh beans may be dried and extracted without loss of activity. It is of no advantage to grind the tissue (thus to wound it severely by mechanical means) before drying and extracting. Of the organic solvents used for this initial extraction, absolute ethyl alcohol was found to be the most effective. The extract which is obtained by extraction with hot alcohol is more *concentrated* in wound hormone than is the corresponding water extract, although a smaller *total amount* of hormone is obtained, even if the alcohol extraction is apparently complete. The alcohol extraction possesses other advantages (4), and has been used in the subsequent procedure for purification.

#### SPECIFICITY OF THE REACTION

A number of pure substances, known to possess physiological activity upon plants, were tested during the course of the work. A partial list of these and other pure substances tried is given in table VI. None of the com-

TABLE VI

PURE SUBSTANCES FOUND TO BE INACTIVE IN THE BEAN TEST

Indole-3-acetic acid	Cystine
Ancurin, (Vitamin B <sub>1</sub> )	Cystine
Lacto-flavin	Alanine
Ascorbic acid	Glycine
1-2-5-6-di-benzanthracene	Methionine
Folliculin	Tyrosin
Pantothenic acid*	Histidine
i-Inositol	Proline
Biotin†	Valine
	Tryptophane
	Leucine
Lecithin	Asparagin
Tannic acid	Aspartic acid
Hesperidin	Glutamic acid
Narinigen	Ornithine
Colchicine	Lysine
Allantoin	Serine
	Betaine

\* Obtained through the generosity of Prof. R. J. WILLIAMS, Corvallis, Oregon.

† Tested on different but reactive beans by Prof. F. W. WENT. Biotin supplied through the courtesy of Prof. F. KÖGL, Utrecht.

pounds elicited any response other than the non-specific or that due to marked toxicity in the higher concentrations. Of particular interest are the following:

*Hetero-auxin*, mentioned by JOST (13) as possessing activity in the bean test. JOST, however, used very high concentrations (1:1000) which apparently were toxic. *Hetero-auxin* was inactive in the present test.

*Aneurin* (vitamin B<sub>1</sub>), a growth factor for other plant tissues (3) but inactive in the bean test.

*Biotin*, *pantothenic acid*, and *i-inositol*, substances of the yeast "bios" group. LAIRD and WEST (15) have found a "bios 2b concentrate" to be active upon their beans. Pure or nearly pure "bios 2b" (biotin, pantothenic acid), however, were inactive in the present test. Prof. R. J. WILLIAMS, furthermore, has very kindly tested our fraction VII-b (below) under a number of conditions and found it to have only negligible "bios" activity.

*Tyrosin*, said by ORSOS (17) to be the active wound hormone principle in the kohlrabi test of HABERLANDT. It was inactive in the present test, as were all of the amino acids tested, both alone and in combination.

*Ascorbic acid*, which, according to HAVAS (10) plays some rôle in abnormal plant growth. It was inactive in the bean test.

*Colchicine*, said by HAVAS (11) to promote abnormal meristematic activity. It was inactive in the bean test.

#### PURIFICATION OF THE WOUND HORMONE

The quantitative test was developed with the particular view of using it as a tool in the isolation and chemical identification of the wound hormone. Each step in the purification was arrived at only after much preliminary groping with this physiological assay as a guide. The evidence for the value of each step cannot be given here. Since a detailed account of the procedure and a discussion of the chemical properties of the products may be found elsewhere (4), only a brief résumé is presented in this paper.

The activities of the various fractions are given below in two ways: (a) that determined from the concentration needed to give a large intumescence (0.30 to 0.70 on the scale of fig. 3), given as activity per mg. relative to the activity per mg. of the initial alcohol extract as unity; and (b) that determined from the minimum concentration giving an intersection point response. This is called the minimum active concentration.

Three separate lots of beans, 100 pounds in all, were extracted. The summary given in table VII is for the extraction of one of these lots, having an initial wet weight of 20 pounds.

It was impossible to increase significantly the activity of VII-b by further extractions or precipitations or even by preparation and distillation of the methyl ester. This free acid concentrate of VII-b is a yellow, extremely hygroscopic, amorphous solid, which is insoluble in ether, chloroform, etc., but readily soluble in water, alcohol, pyridine, etc. It is heat, acid and alkali stable, and contains basic nitrogen although not as a primary amine. The molecular weight as determined by titration, diffusion (4, 12), and by other methods, is in the neighborhood of 220. The ester product of this "free acid concentrate" is a yellow oil which dis-

TABLE VII

RÉSUMÉ OF STEPS IN THE PURIFICATION OF THE ACTIVE PRINCIPLE OF THE  
WOUND HORMONE

PROCEDURE	AMOUNT	ACTIVITY PER MG. RELATIVE TO I-b	MINIMUM ACTIVE CONCENTRATION
I. Extraction of dried beans with absolute alcohol			
a. hot water extract of residue .....		0.0	.....
b. water soluble of alcohol extract	92 gm.	1.0	1: 400
II. Adsorption of I-b with charcoal and elution with pyridine			
a. charcoal filtrate .....	42 gm.	0.0	.....
b. combined eluates .....	52 gm.	1.5	1: 1600
III. Fractional ether precipitation of II-b from pyridine			
a. ether precipitate .....	22 gm.	0.0	.....
b. ether filtrate .....	30 gm.	1.4	1: 4500
IV. Extraction of III-b with ethyl acetate			
a. ethyl acetate insoluble .....	17 gm.	0.3	.....
b. ethyl acetate soluble .....	12 gm.	2.0	1: 12800
V. Conversion of IV-b to its barium salts, alcohol extraction, and regeneration of the free acids			
a. alcohol soluble .....	3.8 gm.	1.6	.....
b. alcohol insoluble .....	6.4 gm.	6.3	1: 36000
VI. Precipitation of V-b with mercuric acetate and subsequent regeneration with H <sub>2</sub> S			
a. mercuric acetate filtrate .....	3.0 gm.	2.8	.....
b. mercuric acetate precipitate .....	1.62 gm.	10.8	1: 100,000
VII. Extraction of VI-b with acetone			
a. acetone insoluble .....	0.216 gm.	10.3	.....
b. acetone soluble .....	1.406 gm.	13.8	1: 100,000
VIII. Preparation and high vacuum distillation of the methyl ester of VII-b			
a. unchanged free acid .....	25% of VII-b	13.1	.....
b. main fraction of ester distillate (after hydrolysis) .....	15% of VII-b	14.4	1: 100,000

tilled well and could be redistilled repeatedly without change of activity. It should be noted that the ester can be tested only after hydrolysis, since it is water insoluble. The products of the three separate extractions gave identical analytical results, the composition of the redistilled ester agreeing approximately with the formula  $C_{11}H_{17}O_4N$ . It seems probable that this product is not far from pure although it has not as yet yielded a single crystalline derivative.



From the 100 pounds of fresh bean which were extracted a total of approximately 500 mg. of distilled ester were obtained. This amount has been too small to permit of further investigations, but it is planned to carry out the extraction upon a larger scale in the near future.

### Discussion

The procedure used for the purification resulted in an enrichment of the active principle of about 250 times over that of the initial alcohol extract, as measured by the minimum active concentration. As measured by the concentration required to produce a given large size of intumescence, the enrichment, however, was only 14 times. Although no portions containing any significant amount of activity were discarded during the procedure, nevertheless the enrichment as measured in this way was much less than the enrichment of material, which was 65 times between alcohol extract and product VII-b. At least two possible explanations suggest themselves:

1. The wound hormone may be continuously altered to a closely related inactive substance.
2. The cooperation of a second substance or substances may be necessary for the production of large intumescences. Such substances should need to be inactive alone, but should augment the effect of the fraction which we have purified.

With regard to the first possibility, it has been impossible to demonstrate that spontaneous inactivation of the wound hormone occurs, although this has been looked for repeatedly. The second possibility also has been borne in mind constantly. At each step of the procedure the two resulting fractions were tested not only separately but *in combination*. In no case was it possible to find any synergistic action of the combined fractions. The free acid concentrate was also tested in combination with other fractions or substances, for example, with charcoal filtrate of fresh extract, fraction II-a, sugar, aneurin, lactoflavin, and hetero-auxin. The slightest evidence for any co-wound hormone activity was never detected.

UMRATH and SOLTYS (22) in a paper which has appeared since the present work was undertaken, have also used the bean test (although in a less quantitative fashion) as an aid for the enrichment of wound hormone. They obtained, from alfalfa, a product having an activity of 1:50,000, or one only slightly less active than that obtained here. Chemically, however, the products differ, that of UMRATH and SOLTYS containing less nitrogen and much more oxygen than that reported here, and apparently being more closely related to the "Mimosa substance" of SOLTYS and UMRATH (21). It seems possible that the apparent difference in composition of the two substances may be due only to a difference in their respective purities.

As we have mentioned in the introduction, HABERLANDT (8) found that

a substance coming from the phloem (lepto-hormone) interacts with the wound hormone in the induction of cell divisions, at least in certain cases such as in the potato tuber. In the case of the bean test, it may be supposed that the lepto-hormone, if it is necessary, may be supplied by the vascular tissue of the pod. Over the range in which response is proportional to applied wound hormone concentration, it is in any case clear that wound hormone rather than lepto-hormone is the limiting factor in the normal bean test. Tests were therefore carried out in which it was attempted to make lepto-hormone the limiting factor. The seed chamber was completely freed of vascular tissue and tests with high concentrations of wound hormone were carried out on the isolated parenchyma. Removal of the vascular tissue actually did reduce the response greatly. If the isolated parenchyma was placed upon agar containing sugar, it responded much more vigorously, although still not as well as the intact controls. One might be tempted to conclude that lepto-hormone, in the case of the bean, consists principally of nutrient sugars. However, the situation is actually more complicated and can be worked out fully only when larger supplies of pure wound hormone are available.

With the isolation of an active wound hormone, even though it may not as yet be in a completely pure state, it is proper to propose a chemical name for the substance. The entire background of the subject from HABERLANDT, in particular, to the present, would seem to make "traumatin" particularly appropriate. It should be stressed again that there may be many wound hormones, that different test objects respond to different chemical substances, and that the present investigation has been concerned with traumatin, *a* plant wound hormone, rather than with traumatin, *the* plant wound hormone.

### Summary

1. The origin and development of the wound hormone concept have been reviewed briefly. Of the several available methods for the demonstration of wound hormone activity, that of WEHNELT (23) was chosen for the present work.

2. A quantitative assay of wound hormone activity has been described. In this test, *size* of the new growth rather than frequency of cell division is measured.

3. The test was shown to be specific for this wound hormone.

4. With the aid of the quantitative assay it was possible to isolate a substance, apparently not far from pure, and possessing typical wound hormone activity. For this substance the name traumatin is proposed.

The authors wish to express their gratitude to Prof. F. W. WENT for

his many suggestions and critical advice during the course of the investigation.

WILLIAM G. KERCKHOFF LABORATORIES OF THE BIOLOGICAL SCIENCES  
GATES AND CRELLIN LABORATORIES OF CHEMISTRY  
CALIFORNIA INSTITUTE OF TECHNOLOGY  
PASADENA, CALIFORNIA

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# EFFECT OF CERTAIN IONS, USED SINGLY AND IN COMBINATION, ON THE GROWTH AND POTASSIUM, CALCIUM, AND MAGNESIUM ABSORPTION OF THE BEAN PLANT

ROBERT L. CAROLUS

(WITH TWO FIGURES)

## Introduction

Almost every season a considerable portion of the acreage of edible podded beans in the Norfolk section, although adequately fertilized with nitrogen, phosphorus, and potash, fails to produce a satisfactory crop because of the fact that some nutritional phenomenon interferes with normal plant growth. As the trouble has occurred in many cases only in certain sections of a field, the poor growth generally could not be attributed to unfavorable climatic conditions. A recent study of magnesium deficiency (3), in which chemical analyses of the plants indicated that in some cases ions other than magnesium altered the absorption and utilization of the magnesium ion by the plant, suggested that some similar phenomenon causing an unbalanced condition in the soil or in the plant might be responsible for the observed unsatisfactory development of the bean crop.

The study of various ions and their interrelations, both in the plant and in the soil, has produced voluminous experimental evidence regarding the rôle of various ions in plant nutrition and plant growth. However, as most of these studies have been conducted with water or sand cultures, the concomitant effects of the soil on these phenomena have not been clearly identified. In the work reported in this paper an attempt will be made to indicate some of the factors, both in the plant and in the soil, that may be partly responsible for the differences in plant response observed in beans grown under conditions produced by the addition of various ions, both singly and in combinations, to the soil. By supplementing the regular tissue analysis of the entire plant with an analysis of the metabolically more active sap of the stems of the plant, a new criterion for determining the effects of the various ionic combinations on both current absorption and total utilization was set up, which may possibly be of material aid in clarifying some of the physiological and chemical relationships existing between the various ions.

## Experimental methods

Seeds of the Bountiful variety of the edible podded string bean (*Phaseolus vulgaris*) were planted September 9 in two-gallon glazed, drained pots filled with a medium composed of one-half soil, classified as a Norfolk fine sandy loam, and one-half washed sand. In colloid and organic matter content this medium probably is comparable to the poorest type of soil in this

section. A test of this medium by the Soils Department, using methods fully described elsewhere (6), indicated a soil reaction of pH 5.1, a good rating for available phosphorus, potassium, and ammonia nitrogen, and a low to fair rating for magnesium. The amounts of the various cations (expressed in terms of their oxides) found in the replaceable state at the end of the third crop, and after a heavy leaching in a pot containing 9 kg. of the unfertilized soil, were 15.9 gm. of potash, 2.8 gm. of calcium oxide, and 1.25 gm. of magnesium oxide.

Each fertilizer treatment, as listed in table I, was applied, after the plants had emerged on September 22, to duplicate pots at a rate equivalent to 500 pounds per acre of a fertilizer analyzing 6 per cent. nitrogen, 6 per cent. phosphoric acid, and 5 per cent. potash. All treatments except number one contained equal amounts of nitrogen and all except numbers one, two, and four contained equal amounts of phosphorus. All cations were applied in equivalent weights. Consequently, those containing two, three, or four cations contained two, three, or four times as much equivalent cation material as those receiving a single cation.

The plants were harvested October 15 when they had reached the blooming stage shown in figures 1 and 2. Determinations of  $K_2O$ ,  $CaO$ , and  $MgO$  in the fresh plant tissue were made as follows: Five-gram portions of finely cut stem tissue taken from plants given each of the treatments were ground in mortars with 20 cc. of 2 per cent. acetic acid and approximately 0.25 gm. of charcoal for 10 minutes, and then filtered at once. Portions of this extract were used in testing for potash with the aid of sodium cobaltinitrite, for calcium oxide with the aid of sodium oxalate, and for magnesia with a weak alcoholic solution of titan yellow. The turbidity developed in the potash and lime test, and the progressive changes in color for different concentrations of magnesia were made the basis of comparison with solutions containing known amounts of potash, lime, or magnesia. Numerous details involved and precautions used in making these tests have already been published (2, 5) and will not be reiterated here.

The roots, leaves, and remainder of the stems from each treatment were dried and subjected to the conventional tissue analyses for magnesia, calcium oxide, and potash (1).

## Experimental results

### GROWTH RESPONSE

In figures 1 and 2, examples of the type and extent of the growth produced under most of the ionic treatments used are shown at the period just prior to sampling for chemical analysis. In the upper row of crocks in figure 1, the general debility and lack of plant growth are evidences that some factor or factors interfered with normal development. Similar conditions of

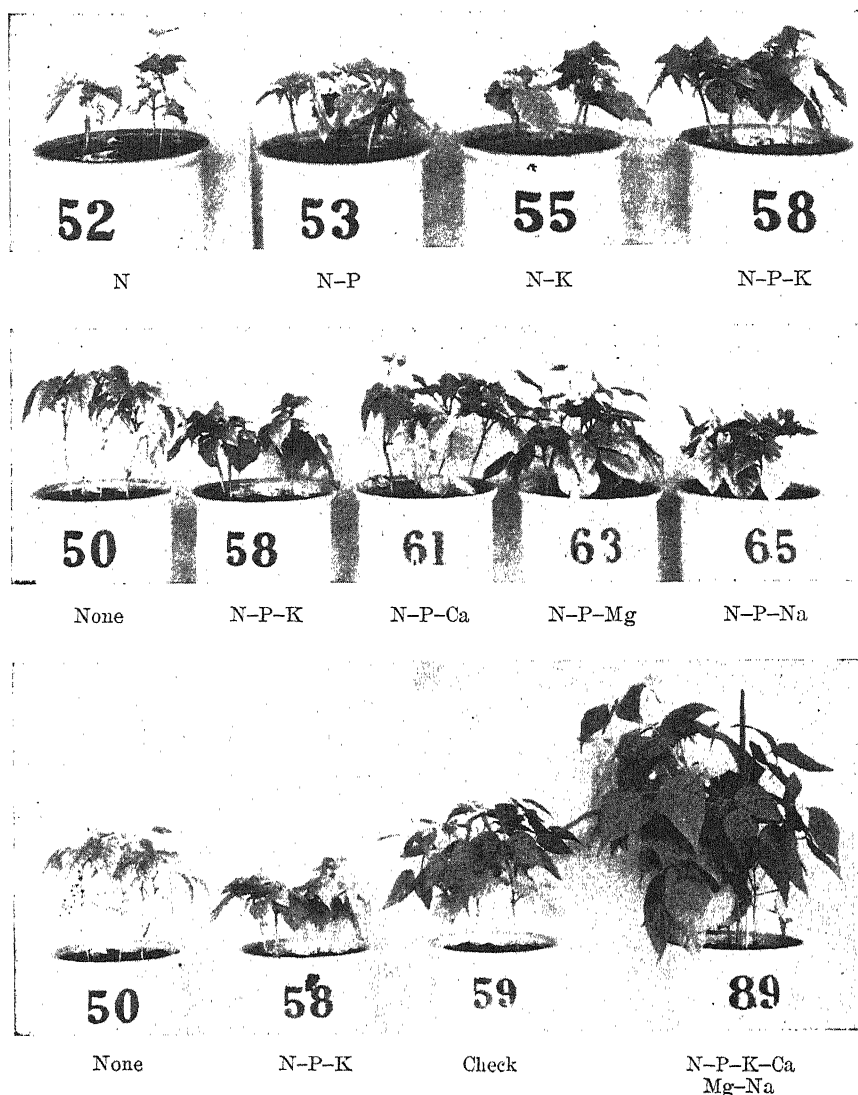


FIG. 1. The influence of certain ions on the growth of beans (*Phaseolus vulgaris*).

chlorosis, dwarfing, and early leaf shedding are often observed under field conditions, and probably are caused by some unbalanced nutrient condition in the soil. In the center row of pots in figure 1, the unfertilized plants (pot 50) are taller than many of those grown in treated pots. However, their dry weight was not equal to that produced by any of the others shown in the center row, but was greater than that produced in pots receiving either N, N-P, or N-K mixtures. The dwarfing of the stems and petioles in the bean

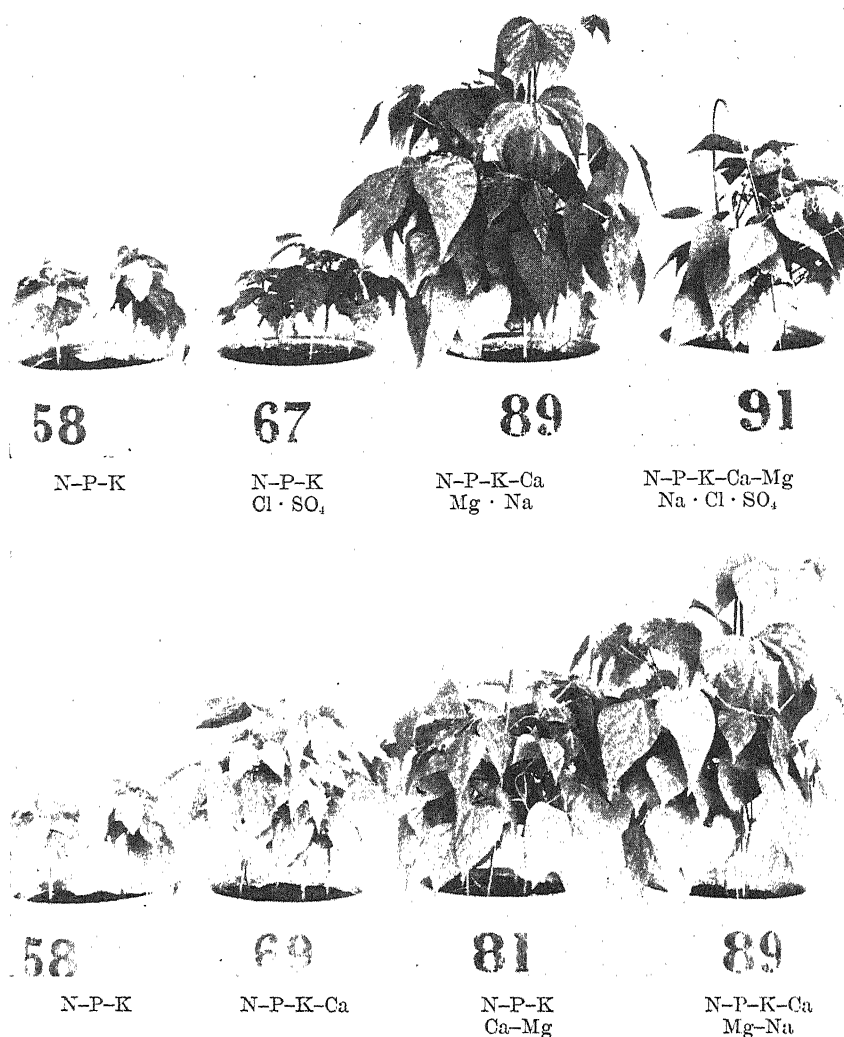


FIG. 2. The influence of certain ions on the growth of beans (*Phaseolus vulgaris*).

plants that received only the sodium cation was a very characteristic symptom of sodium toxicity and has been observed in many other crops grown under similar conditions. In the lower row the effect of a regular commercial fertilizer (pot 50) as contrasted with one that contains only N, P, K, Ca, Mg, and Na ions (pot 89) is shown. For the best growth of beans some of the ions usually included in a regular commercial mixture evidently should be omitted. As the commercial mixture contained considerable sodium from nitrate of soda, an abundance of sulphate from sulphate of ammonia, and all the potash from potassium chloride, the sodium, chlorine, and sulphate ions



probably were partially responsible for some of the differences in growth that were observed. In the upper series in figure 2 this idea is substantiated for the chlorine and sulphate ions. Pot 91 received the same cations as were used in pot 89, but, in addition, received some chlorine and sulphate ions which probably were responsible directly or indirectly for the observed differences in growth.

In the lower series of pots in figure 2 the detrimental effect of cation unbalance is readily observed. Growth was more responsive to fertilizer containing all four cations than to any mixture from which one was omitted. Although beans respond positively to a mixture of all four cations, the potato,

TABLE I

CHEMICAL COMPOSITION OF FERTILIZERS USED

TREATMENT NO.	DESCRIPTION	CONSTITUENTS AND AMOUNTS APPLIED TO EACH POT (U. S. P. CHEMICALS USED)
1	None	None
2	N	$\text{NH}_4\text{NO}_3$ , 1.39 gm.
3	N-P	$\text{NH}_4\text{NO}_3$ , 1.12 gm. + $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.79 gm.
4	N-K	$\text{NH}_4\text{NO}_3$ , 1.04 gm. + $\text{KNO}_3$ , 0.88 gm.
5	N-P-K	$\text{NH}_4\text{NO}_3$ , 0.77 gm. + $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.79 gm. + $\text{KNO}_3$ , 0.88 gm.
6	Check	A 6-6-5 (2% MgO) neutralized fertilizer (commercial)
7	N-P-Ca	$\text{NH}_4\text{NO}_3$ , 0.77 gm. + $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.79 gm. + $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1.01 gm.
8	N-P-Mg	N and P (as above) + $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1.10 gm.
9	N-P-Na	N and P (as above) + $\text{NaNO}_3$ , 0.74 gm.
10	N-P-K-Cl-SO <sub>4</sub>	N and P (as above) + KCl, 0.32 gm. + $\text{K}_2\text{SO}_4$ , 0.37 gm.
11	N-P-K-Ca	$\text{NH}_4\text{NO}_3$ , 0.42 gm. + $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.79 gm. + $\text{KNO}_3$ , 0.88 gm. + $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1.01 gm.
12	N-P-K-Mg	N and P (as above) + $\text{KNO}_3$ , 0.88 gm. + $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1.10 gm.
13	N-P-K-Na	N, P, and K (as above) + $\text{NaNO}_3$ , 0.74 gm.
14	N-P-Ca-Mg	N and P (as above) + $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1.01 gm. + $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1.10 gm.
15	N-P-Ca-Na	N, P, and Ca (as above) + $\text{NaNO}_3$ , 0.74 gm.
16	N-P-Mg-Na	N and P (as above) + $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1.10 gm. + $\text{NaNO}_3$ , 0.74 gm.
17	N-P-K-Ca-Mg	$\text{NH}_4\text{NO}_3$ , 0.08 gm. + $\text{NH}_4\text{H}_2\text{PO}_4$ , 0.79 gm. + $\text{KNO}_3$ , 0.88 gm. + $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1.01 gm. + $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1.10 gm.
18	N-P-K-Ca-Na	N, P, K, and Ca (as above) + $\text{NaNO}_3$ , 0.74 gm.
19	N-P-K-Mg-Na	N, P, and K (as above) + $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1.10 gm. + $\text{NaNO}_3$ , 0.74 gm.
20	N-P-Ca-Mg-Na	N and P (as above) + $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1.01 gm. + $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1.10 gm. + $\text{NaNO}_3$ , 0.74 gm.
21	N-P-K-Ca-Mg-Na	$\text{NH}_4\text{NO}_3$ , 0.27 gm. + $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.94 gm. + $\text{NaNO}_3$ , 0.15 gm. + $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1.01 gm. + $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1.10 gm. + $\text{KNO}_3$ , 0.88 gm.
22	N-P-K-Ca-Mg-Na-Cl-CO <sub>4</sub>	$\text{NH}_4\text{NO}_3$ , 0.62 gm. + KCl, 0.32 gm. + $\text{K}_2\text{SO}_4$ , 0.37 gm. + $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.94 gm. + $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 1.01 gm. + $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 1.10 gm. + $\text{NaNO}_3$ , 0.15 gm.

as previously reported (4), is adversely affected by mixtures of equivalent weights of K, Ca, Mg, and Na. Therefore, the results obtained with beans probably cannot be extended to apply generally to all crops.

#### METHODS OF EVALUATING THE CHEMICAL ANALYSES

The growth, expressed in terms of dry weight, and the  $K_2O$ ,  $CaO$ , and  $MgO$  composition of the plants from all treatments except nos. 18, 19, and 20 are found in tables II and III. The treatments are arranged from left to right in the order of their influence on the total dry weight of foliage produced. All figures expressing the chemical composition are averages of duplicate determinations, made on duplicate samples in those cases where the material was sufficient to allow such a procedure. Determinations of nitrogen and phosphorus were made also, but were not included in order to simplify the tabular material and because in most cases the results indicated that nitrogen and phosphorus determinations were of no significance.

In discussing the absorption of the cations occurring in the plants fertilized with the different ionic combinations, as indicated by the figures in the tables, the percentage of the cation in the dried plant will be considered as a measure of the relative ability of the plant to utilize that material under the conditions of the experiment. The presence of a cation, expressed in terms of parts per million in the extracted sap of the stem of the freshly harvested plant, will be considered as reflecting the absorptive ability of the plant under each of the treatments, or as partially indicative of the availability of the cation to the plant. However, it also reflects the ability or inability of the plant to utilize the absorbed material when considered in relation to the total amount of a given cation that was absorbed. For example, a high content of a cation in p.p.m. in the stem sap may indicate rapid and unhindered absorption or may indicate an accumulation resulting from low total utilization. In turn, low total utilization may be considered as due to retarded growth or to the inability of the plant to build the particular cation into differentiated structures or substances. If the total utilization is not proportional to the growth made, the latter may be considered as the factor responsible for the low total utilization. From a comparative study of a cation in the plant in terms of percentage of the dry weight, p.p.m. in the stem sap, and total absorption per unit, it should be possible to determine whether or not the phenomena observed are due to the effect of the treatment on absorption, on utilization, or on both.

#### ABSORPTION OF CATIONS

In order to identify and clarify some of the reasons for the differences in growth found (figs. 1, 2) resulting from the use of the N-P-K-Ca-Mg-Na treatment as compared with the N-P-K treatment, an outline is given

TABLE II

EFFECT OF CERTAIN CATIONS, USED SINGLY AND IN COMBINATION, ON THE GROWTH AND POTASSIUM, CALCIUM, AND MAGNESIUM ABSORPTION OF BEANS RECEIVING A UNIFORM NITROGEN AND PHOSPHORUS TREATMENT

TREATMENTS	5	8	9	7	14	13	16	12	15	11	17	21
	K	Mg	Na	Ca	Ca Mg	K Na	Mg Na	K Mg	Ca Na	K Ca	K-Ca Mg	K-Ca Mg-Na
Dry eight foliage (gm. per pot) .....	2.2	2.3	2.6	2.7	3.0	3.6	4.2	4.3	4.4	5.3	8.3	12.3
Potassium (K <sub>2</sub> O):												
Percentage in foliage												
(dry weight basis) .....												
P.p.m. in stem sap* .....	5.9	2.0	1.9	2.0	2.0	4.9	1.7	5.6	1.7	5.2	4.6	4.0
Mg. absorbed per pot .....	13500	2500	2000	3000	400	5200	800	6000	2000	6500	5700	4200
Percentage of applica-	132	46	50	54	60	176	72	241	75	276	382	492
tion absorbed by plants	33	—	—	—	—	44	—	60	—	68	95	122
Calcium (CaO):												
Percentage in foliage												
(dry weight basis) .....												
P.p.m. in stem sap* .....	1.5	1.6	1.9	2.6	1.9	1.6	1.5	1.3	2.8	2.3	2.1	1.9
Mg. absorbed per pot .....	300	375	650	1800	400	650	550	350	2000	750	650	400
Percentage of applica-	33	37	50	70	57	58	63	56	123	122	174	234
tion absorbed by plants	—	—	—	29	24	—	—	—	51	51	72	97
Magnesium (MgO):												
Percentage in foliage												
(dry weight basis) .....												
P.p.m. in stem sap* .....	1.3	2.0	1.1	1.0	1.9	1.2	2.2	1.6	1.4	1.1	1.8	1.8
Mg. absorbed per pot .....	50	650	900	1000	1100	110	125	200	650	125	600	525
Percentage of applica-	29	46	29	27	57	43	93	69	62	58	149	222
tion absorbed by plants	—	28	—	—	35	—	57	42	—	—	92	136

\* Soluble in a 2 per cent. acetic acid extract of the stems, and based on the fresh weight of the stem tissue.

in table II of the effects of each of the more significant combinations of the four cations on the  $K_2O$ ,  $CaO$ , and  $MgO$  contents of the plants. In this series of treatments the nitrogen and phosphorus applications remained constant and equivalent weights of each cation were used in making up the different treatments. The first treatment (N-P-K) resulted in a larger accumulation of  $K_2O$  in the sap of the stems than was found in any of the other treatments. The addition of potassium had a more marked influence on potassium absorption than the addition of either calcium or magnesium had on their own absorptions. The potash content of the plant stem sap from the treatment in which potassium was the only cation used was 13,500 p.p.m. or over twice the amount found in the sap of plants in any other treatment. Probably this extremely large concentration of potassium was the factor that was responsible for the poor growth made with the N-P-K treatment. This amount of potassium probably was toxic, due to the fact that its presence in the plant sap in such a concentrated soluble proportion inhibited the absorption or utilization of sufficient calcium and magnesium to permit normal growth. Calcium was less detrimental to growth and absorption than any of the other cations used singly with nitrogen and phosphorus.

Of the six combinations of two cations, the K-Ca combination was most efficient in producing maximum growth and promoting maximum absorption of both potassium and calcium, but one of the least efficient in promoting the absorption of magnesium. The Ca-Mg treatment checked both absorption and utilization of potassium, as indicated by a low  $K_2O$  content in p.p.m. in the stem sap and by a low total potash absorption by the plant. The K-Na treatment resulted in a lower absorption and utilization of potassium than occurred in any of the other potassium treatments. In treatments numbered 9, 16, and 15, in which sodium was present, a low potassium absorption and utilization were also found. The K-Na combination was also detrimental to magnesium absorption and utilization. The Mg-Na treatment was detrimental to adequate absorption of potassium and magnesium and to the utilization of potassium and calcium. In this treatment the highest concentration of magnesium (2.2 per cent.) was found in the plant, but the absorption rate as indicated by a concentration of 125 p.p.m. on the stem sap was checked by some external factor, probably a lack of antagonism between the magnesium and sodium ions (7). In the K-Mg combination the low absorption and utilization of calcium probably is most responsible for the resultant lack of growth, but the detrimental influence of potassium on magnesium absorption as observed in the N-P-K treatment probably also existed. In the Ca-Na combinations the antagonistic effect of the sodium ion on the calcium ion resulted in a greater absorption of calcium, indicated by a concentration of 2000 p.p.m. of  $CaO$  in the stems, and also in a greater utilization, indicated by a  $CaO$  content of 2.8 per cent. of the dry weight of the plant. However,

the detrimental influence of sodium on potassium absorption overshadows any beneficial effect of calcium on potassium absorption, and results in a low potassium absorption, which probably was the factor responsible for poor growth. The K-Ca combination was the best double cation combination used, because Ca did not exert a detrimental effect on potash absorption and overcame the detrimental effect of potassium on calcium absorption. The nutrient limiting growth in this treatment was probably magnesium.

The addition of magnesium to the K-Ca combination increased magnesium absorption to a satisfactory degree and, because its detrimental influence upon potassium and calcium absorption was held in check by the inclusion of these two ions, resulted in satisfactory growth.

The addition of the sodium ion to the K-Ca-Mg combination necessitated the use of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  instead of  $\text{NH}_4\text{H}_2\text{PO}_4$  in order that the nitrogen content could be maintained at a constant level for all treatments. The increase in growth produced with this combination over that produced with the K-Ca-Mg treatment was not associated with any beneficial effects on the absorption of potassium, calcium, or magnesium. As the phosphorus content was increased from 0.52 to 0.65 per cent. of the dry weight of the plant by including the sodium cation, increased phosphorus availability and utilization were probably responsible for the increased growth produced. Whether or not the sodium ion as such, or the form in which the phosphorus ion was used, was responsible for the increased phosphorus absorption, was not determined. However, in a previous work (4) sodium, when used in the same formulation with K-Ca-Mg, was very detrimental to the growth of potatoes. Hence it would seem, from a comparison of the behavior of potatoes and beans, that beans are the more tolerant of the sodium cation when it is associated with the other cations.

#### DISCUSSION OF CATION ABSORPTION

In beans, the potassium cation, when added to the soil, produced such a dominant effect on potassium absorption that it was able to counteract the detrimental effect of any of the other cations on its absorption by the plant. However, neither the calcium nor the magnesium cations were able to exert such a dominant effect on their own absorptions when they were combined with other cations that tended to interfere with their own absorptions.

The concentration of potassium in the stems of the plants indicated a high degree of negative correlation with growth. For example, treatments numbered 5, 13, 12, 11, 17, and 21 had all received an equal quantity of potassium and the stem sap of the plants from these treatments ranged in p.p.m. of  $\text{K}_2\text{O}$  from 13,500, in the case of those exhibiting the poorest growth, down to 4200 p.p.m., in the plants exhibiting the best growth. From these observed phenomena we may be safe in concluding that nothing interfered with potas-

sium utilization or absorption to any marked extent. Had this not been the case, the concentration of  $K_2O$  in the sap would have dropped below the optimum with the increased utilization or lack of absorption of potassium by the plant, or, would have increased above that found at minimum growth.

Sodium had a marked stimulating effect on calcium absorption, while potassium exerted little or no influence on calcium absorption and magnesium had a marked detrimental effect on the absorption of this cation. These influences were quite evident when the  $CaO$  content of the sap from treatments 7, 14, 15, 11, 17, and 21 were compared. In the  $Ca$  treatment 1800 p.p.m. of  $CaO$  was found in the stem sap. However, when magnesium was added, the  $CaO$  content of the sap dropped to 400 p.p.m. although growth did not increase more than 10 per cent. When sodium was substituted for magnesium, the  $CaO$  content of the sap increased to 2000 p.p.m. under conditions in which the growth also increased over 50 per cent. However, as the  $CaO$  content of the sap from the plants of treatments 11, 17, and 21 showed some indication of negative correlation with growth, it is evident that potassium when applied in conjunction with calcium was able to minimize the negative influence of magnesium and the positive influence of sodium on calcium absorption.

Potassium, calcium, or sodium had a greater influence on magnesium absorption by the plant when used in conjunction with magnesium than the magnesium cation had on its own absorption. Although magnesium was detrimental to the absorption of calcium, calcium facilitated magnesium absorption and utilization. This phenomenon may be partly responsible for the fact that dolomitic limestones are generally as efficient correctives for a condition of magnesium deficiency in the soil and plant as the more soluble magnesium salts (3). Both sodium and potassium prevented normal magnesium absorption and potassium interfered also with its utilization in the plant. These phenomena were evident when the magnesium contents of the plants in treatments 8, 14, 16, 12, 17, and 21 were compared. With magnesium alone, the  $MgO$  content of the stem sap was 650 p.p.m., but when calcium was added it increased to a probable toxic concentration of 1100 p.p.m. even though a 25 per cent. increase in growth was made. The  $Ca-Mg$  combination was found to be toxic to the potato plant also (4), manifested by a purpling of the central portions of the leaf. However, when sodium was associated with magnesium, the  $MgO$  content of the sap fell to 125 p.p.m. This phenomenon probably was due to a lack of absorption brought about by a lack of antagonism. MILLER (7) reports that an antagonistic influence between the sodium and magnesium ions has not been demonstrated. This phenomenon may be due in part, however, to the rapid utilization of magnesium. Again, when potassium was associated with magnesium, the  $MgO$  content of sap dropped to 200 p.p.m. However, when calcium was associated

with the K-Mg combination, its beneficial influence on absorption counteracted the detrimental effect of potassium, and both normal absorption and normal utilization resulted.

#### EFFECT OF VARIOUS ANIONS ON CATION ABSORPTION

Although the various cations exerted marked influences on growth, as indicated by a difference of 10.1 gm. in the dry weight produced per pot between the N-P-K and the N-P-K-Ca-Mg-Na treatments, some of the anions were also of physiological significance, as shown by the analyses in table III.

The use of nitrogen alone (treatment no. 2) did not depress calcium or magnesium absorption below that occurring under optimum growth conditions. However, a slight depression in potassium absorption and a large increase in nitrogen absorption (4540 p.p.m. of soluble N) as compared with a nitrogen absorption under optimum growth conditions of 830 p.p.m. indicated that the concentration of these two materials probably was responsible for the poor growth. Additions of either  $\text{PO}_4$  or K to the  $\text{NO}_3$  treatment depressed Mg absorption to a point where it became one of the growth-limiting factors. Potassium with nitrogen increased potash absorption to a concentration where it again probably caused the previously mentioned unbalanced or toxic condition in the plant. In the unfertilized treatment a striking increase in the absorption of calcium and magnesium over that of the previously discussed treatments occurred, but the absorption of potassium fell below that for the plants in the other treatments. Poor growth in the unfertilized pots probably was due chiefly to a lack of nitrogen as indicated by a concentration of 81 p.p.m. of soluble N in the sap of the stems. However, it was observed in the N-K treatment that by the addition of nitrogen and potassium alone growth was depressed below that found when no fertilizer was used, which would seem to indicate that any fertilizer applied must have a rather definite ionic balance.

The commercial fertilizer treatment produced a larger amount of dry weight than any of the combinations of N, P, or K, but it was materially smaller than that produced with the N-P-K-Ca-Mg-Na treatment (no. 21). The absorption and utilization of both potassium and calcium approximated that found in the N-P-K-Ca-Mg-Na treated plants, but the decreased utilization of magnesium, as shown by a magnesia composition of 1.2 per cent. as compared with 1.8 per cent. in the optimum treatment, indicated that some phenomenon was still hampering the normal functioning of the plant.

After comparing the dry weight produced in treatment no. 21 with that of no. 22, it must be concluded that either or both chlorine and sulphate ions were partially responsible for the poor growth realized with a commercial fertilizer treatment containing large amounts of both of these ions. A toxic





or unbalanced anion condition resulting from the presence of Cl or SO<sub>4</sub> ions in treatment no. 22 is responsible for the difference in the growth produced between treatments 22 and 21, because the potassium, calcium, and magnesium utilization and absorption in both treatments were fairly comparable. In treatment 10 in which Cl and SO<sub>4</sub> ions were added to the N-P-K treatment, an increased growth was realized. The fact that both had considerably less total dry weight than the N-P-K-Ca-Mg-Na treatment indicated that in this comparison some other factor or factors were more detrimental to growth than the presence in the soil of either Cl or SO<sub>4</sub>. In fact, the addition of Cl and SO<sub>4</sub> ions to the N-P-K mixture caused a slight increase in plant growth, which indicated that these two ions are of value in partially counteracting a certain type of unbalance, but that after this condition is corrected by the addition of Ca, Mg, and Na ions, their own toxic influence becomes one of the factors limiting the growth of beans.

#### SUMMARY OF THE INFLUENCE OF VARIOUS IONS ON CATION ABSORPTION AND UTILIZATION

The influences of most of the common ions included in a normal mixed fertilizer on the absorption and utilization of the three cations, potassium, calcium, and magnesium, by bean plants, are summarized in schematic form in table IV. In this summary the mean influence of the ions in all combinations, considered in relation to the optimum treatment N-P-K-Ca-Mg-Na,

TABLE IV

SUMMARY OF THE INFLUENCE OF VARIOUS IONS ON CATION ABSORPTION AND  
UTILIZATION BY BEANS

Ion	NO <sub>3</sub> NH <sub>4</sub>	PO <sub>4</sub>	Cl SO <sub>4</sub>	K	Ca	Mg	Na
K <sub>2</sub> O absorption .....	-	-	0	+++	0	--	-
K <sub>2</sub> O utilization .....	-	-	0	+++	0	-	--
CaO absorption .....	0	0	0	-	++	--	+++
CaO utilization .....	0	0	0	-	++	-	++
MgO absorption .....	0	-	0	---	+++	0	--
MgO utilization .....	-	-	0	--	+	0	+

Key: --- very detrimental (probably resulting in a deficiency); -- detrimental; - slightly detrimental; 0 probably of no influence; + slightly beneficial; ++ beneficial; +++ extremely beneficial (probably resulting in a toxicity).

was used as a basis in estimating the effects listed. As mentioned earlier in the paper, 15.9 gm. of replaceable K<sub>2</sub>O, 2.8 gm. of replaceable CaO, and 1.25 gm. of replaceable MgO per pot were found in the unfertilized soils. Some differences in the influence of each ion would probably be observed under a different condition of base saturation. However, as the bean crop was the third and last of a series grown on this soil medium, very likely only small

portions of the total exchangeable cations were influencing actively the phenomena observed. This contention would seem to be substantiated by the fact that on the unfertilized soil 15.9 gm. of replaceable  $K_2O$  had no apparent detrimental influence on calcium and magnesium absorption, while the addition of 0.40 gm. of  $K_2O$  in a soluble form had a most detrimental effect on the absorption of these two ions.

### General summary and conclusions

1. The present trend in the fertilizer industry toward the elimination of sodium, chlorine, and sulphur, a reduction in the amount of calcium, and the inclusion of magnesium in increasing amounts, warranted a study of the effects of these materials on growth, cation absorption, and utilization.

2. On a soil medium that in the past had received considerable fertilizer under field conditions, bean plants without additional fertilizers were able to absorb sufficient calcium and magnesium, but did not make a normal growth, probably because of a lack of nitrogen, phosphorus, and potash.

3. When an N-P-K mixture was added to the above mentioned unfertilized soil the intake of magnesium and calcium was depressed to such an extent that they became two of the limiting factors to optimum growth.

4. The addition of the potassium cation to a soil stimulated the potash absorption by bean plants to a greater extent than the addition of calcium or magnesium cations stimulated their absorptions by the plant. None of the other cations employed had any very pronounced beneficial or detrimental influence on potassium absorption when the potassium ion was included in the fertilizer treatment. However, the addition of potassium when combined with magnesium materially reduced the absorption of both calcium and magnesium, and when combined with calcium reduced magnesium absorption. It did not effect an increased absorption of any other ion.

5. The addition of calcium to a soil not only increased calcium absorption, but in all cases facilitated a large magnesium absorption, and in some cases was slightly beneficial to potassium absorption.

6. The absorption of the magnesium cation by bean plants was the most difficult phenomenon to control, because the addition of the magnesium cation to a soil had less influence than that of any of the other cations used on magnesium absorption.

7. The sodium ion had a beneficial effect on calcium absorption and utilization, and was beneficial to magnesium utilization, but in most cases was slightly detrimental to the absorption of potassium and extremely detrimental to the absorption of magnesium.

8. The only method of obtaining an adequate absorption of all three cations was to include them in the fertilizer mixture. Under these conditions the addition of the sodium ion to this combination was not detrimental to the growth of beans and possibly was of some direct benefit.

9. The anions  $NO_3$ ,  $PO_4$ ,  $Cl$ , and  $SO_4$  had no detrimental influence on

calcium intake and utilization, but the  $\text{NO}_3$  and  $\text{PO}_4$  anions had a slight detrimental effect on potassium absorption and probably a slight detrimental effect on magnesium utilization.

10. The use of rapid chemical methods in analyzing the sap of plant stems was found to be of value in determining whether deficiencies of the cations were due to a lack of absorption or to a lack of utilization by the plant.

11. From this work it may be concluded that if plants showing *poor* growth have a higher content of a given nutrient (as determined by p.p.m. in stem sap) than similar plants showing a *superior* growth, something is interfering with the proper utilization of that nutrient *after* its absorption by the plant. This is the situation in the potassium series. However, if the plant sap of plants of *superior* growth has a higher content of a given nutrient than that of plants showing *poor* growth, the cause is likely to be lack of ability to absorb this nutrient in the case of the stunted plants. This occurs in the magnesium series. Failure to absorb may be due to a deficiency of the nutrient in an available state, or to some absorption phenomenon such as lack of antagonism.

12. The results in these tests should not be construed as applying to all plants, as work not far enough advanced to report at this time has indicated that other plants do not respond in a manner similar to that herein reported for beans.

VIRGINIA TRUCK EXPERIMENT STATION  
NORFOLK, VIRGINIA

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# CHEMICAL AND SPECTROSCOPIC ANALYSIS OF PHLOEM EXUDATE AND PARENCHYMA SAP FROM SEVERAL SPECIES OF PLANTS

CARLETON A. MOOSE

(WITH THREE FIGURES)

## Introduction

A problem which has occupied the attention of botanists for many years is that of the transport of organic and inorganic substances in plants particularly with respect to the tissues active in translocation. Since there is still some doubt concerning the transport of inorganic constituents in the phloem, it would seem desirable in this connection to make an analysis of phloem exudate to see what materials may be present therein. The exudate used in these analyses is probably restricted to phloem tissues alone as methods of collection were used which have been employed by previous investigators (HARTIG, 12, MÜNCH, 20, and CRAFTS, 3). JAMES and BAKER (15), in working with maple claim that the sap exuded did not come from the phloem alone but also from the living cells particularly of the cambial zone. Their work, however, was done in England during the winter months when the cambial activity must have been slight. GROSSENBACHER (10), EAMES and MACDANIELS (7) and other investigators have abundant evidence to indicate that the maximum cambial activity occurs at the time of elongation of the shoots which is in the early part of the summer for the species included in this study. Hence the chance of exudation from the cambium along with the phloem exudation is slight, since the exudate for the present study was collected during the months of August and September. In this connection PRIESTLEY (23) reports that if the bark is stripped from the new wood in May or June and a cut made across the differentiating tissues of soft wood, a fine spray of liquid spurts several inches from the cut while a similar cut in hard wood results in a gentle oozing with no spray. These observations tend to support the idea that exudation is not limited to the phloem alone during the active growing season. They do not indicate, however, that the exudate later in the season should be from tissues other than the phloem.

PFEIFFER (22) has made determinations on the osmotic concentrations of sieve tube sap of *Quercus rubra* by a cryoscopic method and finds a range of values from 15.0 atm. in August to 21.1 atm. in November. MÜNCH (20) gives values for osmotic pressure of sieve tube sap of *Quercus rubra* varying from 20.9 atm. to 23.7 atm. and values from 25.8 atm. to 34.3 atm. for sieve tube sap of *Robinia pseudo-acacia*.

KRAUS (16) and ZACHARIAS (26) have made some qualitative tests on the

sieve tube sap of *Cucurbita pepo*. A quantitative analysis of phloem exudate has not been made, however, previous to this work probably because of the difficulties attendant upon collection of sufficient quantities of the exudate to permit an analysis by methods other than microchemical or spectroscopic. It has been the purpose of this work to analyze quantitatively the phloem exudate and parenchyma sap with respect to the following: pH, total sugars, reducing sugars, calcium, magnesium, potassium, lithium, nitrate nitrogen, and total nitrogen, and to make a qualitative spectroscopic analysis for any other inorganic elements that might be present in the sample. The species studied were *Fraxinus americana*, *Fraxinus pennsylvanica* var. *lanceolata*, *Platanus occidentalis*, *Robinia pseudo-acacia*, and *Cucurbita maxima*. Quantitative spectroscopic methods of analysis which will be described later were used wherever possible because of their efficacy in dealing with small amounts of material.

The method of collecting the phloem exudate in the woody plants is one that has been used by MÜNCH (20). A slit in the trunk about two inches long at an angle of about 45° with the horizontal made with a sharp knife was found to yield the best exudation. The cut was made into the active phloem tissues from which would exude amounts of sap varying from a few drops to 2 or 3 cc. depending upon the species and the length of the cut. As the drops of liquid appeared, usually near the base of the cut, they were picked up with a glass capillary tube fitted with a small rubber bulb and transferred immediately to hard glass phials which contained a few cc. of toluene as a preservative. Several cuts were made at a time and the amount usually collected after two or three hours was about 10 cc.

The time of day seemed to be a factor in governing the amount of flow, the largest quantities being obtained in the afternoon. Bright sunny days usually gave better results than cloudy cold ones, although on a few occasions the flow from *Robinia pseudo-acacia* was good on cloudy days. On days when the transpiration stream was rapid a cut deep enough to penetrate to the xylem tissue would result in the exudate being sucked into the transpiration stream often with an audible sucking sound.

HARTIG (12) and MÜNCH (20) report that a second cut within from one to five meters below, and for a shorter distance above, an original cut would yield no flow. However, in both species of *Fraxinus*, and in *Platanus occidentalis* the presence of a second cut either above or below the first cut did not appreciably affect the flow from the first cut unless the second cut was within 5 or 6 cm. on either side of the first. The flow from the second cut would then be as good as that from the first. The second cut would give a poor flow when too close to a cut above, and a second cut would diminish the flow from the first cut when too close above the first. A distance of 10 or 12 cm., however, seemed to make no difference except in *Robinia pseudo-acacia*

where a second cut would yield no flow unless displaced laterally with respect to the first. In this species a second cut even 1 or 2 meters directly above or below a first cut would yield no flow.

In the case of *Cucurbita maxima* the method of collection of phloem exudate was that used by CRAFTS (3). The plants were grown in the greenhouse, and when the fruits had developed to a length of about 8 or 10 cm. they were cut from the vine leaving about 2.5 cm. of peduncle attached to the fruit. A drop of crystal clear exudate coming from the phloem proper and from the ectocyclic and endocyclic sieve tubes which are in the position of a latex system would appear on this cut end of the peduncle. This was allowed to drop into a small porcelain crucible containing a few cc. of toluene. About two drops of exudate would be obtained before the flow stopped, but the flow could be started again by cutting a short section from the end of the peduncle. This process could be repeated until the peduncle had been cut back to the fruit and a total of about ten drops of exudate obtained. Fifteen or twenty fruits would give a sample sufficient for several of the analyses.

The parenchyma sap was obtained by freezing, at  $-15^{\circ}$  C., leaf tissue contained in large test tubes in an alcohol bath cooled by a G. E. cooling unit, and then pressing out the sap by means of an hydraulic press. Pressures of 10,000 pounds per square inch were used, and the tissue was subjected to this pressure for five minutes. Samples of the sap were kept in the refrigerator until ready for use.

### Analytical methods

The pH values of all samples were obtained immediately after collection by use of the quinhydrone electrode method described in Leeds-Northrup Co., Bulletin no. 3.

The sugar analyses were made as soon after collection as convenient, in order to minimize the error due to chemical changes. All samples were shaken well with toluene and kept in the refrigerator until analysis was begun. The toluene prevented any changes due to the growth of organisms but had no effect on any enzymes which might have been present in the exudate. It is not certainly known that any enzymes were present. The modified SHAFFER (24) method of sugar analysis was used since the colorimetric determination of the  $\text{Cu}_2\text{O}$  is very accurate when small quantities of material must be used.

The modification of the spectroscopic method for the determination of potassium as developed by HICKS (13) will be described in some detail. The absence of sensitive arc lines of potassium when small amounts of the element are involved prevents the use of the arc and spectrograph in quantitative analyses for this element; but there is a line in the flame spectrum of potassium (wave length 7669 Å.) which is extremely sensitive and which shows

up when the element is present in small quantities. The duration of this line when potassium is introduced into the flame is proportional within limits to the amount of the element present in the flame. The duration of the line is measured by direct visual observation, the observer starting a stop watch upon appearance of the line and stopping the watch upon the line's disappearance. The line flashes into view suddenly and fades out as rapidly so that the timing can be accurate to within 0.1 second. The spectrometer used was the ordinary three arm type described by BALY (1). The Bunsen flame of illuminating gas was standardized as far as possible as to position with respect to the slit of the instrument, height, and mixture. The substance was introduced into the flame in the following manner. A platinum wire was coiled into a small spiral of five turns, 2 mm. in diameter, in such a way that the turns of wire were not in contact in any part of the coil. This coil, once wound, was not disturbed in shape or size during the entire series of tests, and when it was dipped into a liquid it would always pick up and hold the same size drop thus assuring the same amount of a given concentration of material in the flame each time an observation was made. After the platinum wire was dipped into the solution it was held near another flame until the liquid had evaporated leaving only a solid incrustation on the wire which was then introduced into the flame and observed. This evaporation prevented loss of material due to spattering, which would result if a drop of liquid were suddenly thrust into the Bunsen flame.

Standard solutions of potassium chlorate<sup>1</sup> containing potassium in amounts of 0.32, 0.64, 0.96, 1.28 mg./cc. were made from recrystallized salt (Baker's analyzed chemicals). The duration of the 7669 Å. line was observed for each of these concentrations, the average time of ten observations in each case being taken as the final result.

TABLE I  
RELATION BETWEEN CONCENTRATION OF SOLUTION AND DURATION OF THE  
POTASSIUM LINE 7669 Å.

TIME	CONCENTRATION OF POTASSIUM
<i>sec.</i>	<i>mg/cc</i>
2.00	0.16
2.40	0.32
2.60	0.48
2.80	0.64
3.00	0.80
3.30	0.96
3.72	1.28

<sup>1</sup> It was later learned that the use of perchloric acid would have given more accurate results, with potassium chloride rather than potassium chlorate taken as standard.



Table I shows the results of such a series of observations and figure 1 shows the curve of concentration of solution plotted against duration of line.

It is necessary now to observe the duration of line 7669 Å. for the various samples of exudate and sap and from the observed times read from the curve the corresponding values of the concentration of potassium. Because the presence of sugars and other organic materials in the exudate prevented the

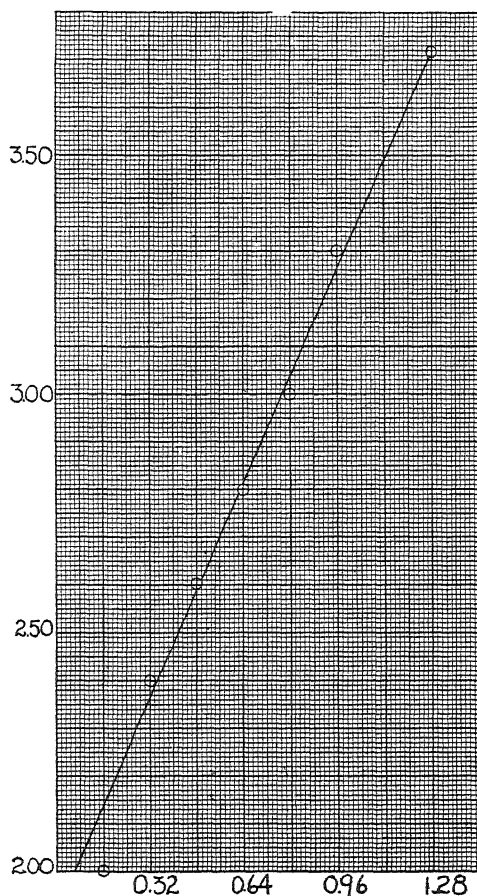


FIG. 1. Relation between concentration of solution and duration of potassium line 7669 Å.

use of the material directly, a known volume of the exudate was evaporated to dryness and ashed in a muffle furnace at a temperature not exceeding 450° C. The temperature was kept comparatively low in order to prevent any loss of the calcium, magnesium or potassium compounds during the ashing process. The ash was dissolved in hydrochloric acid<sup>2</sup> and brought

<sup>2</sup> See footnote p. 368.

back to the original volume with distilled water. This solution was then given five tests and the average time for the five taken as final. Since these values seldom varied by more than 0.2 sec., five observations were considered sufficient. The corresponding concentrations for each sample were recorded. During a series of observations on unknowns the system was checked from time to time by making observations on one or more known concentrations, and at the beginning of a new series of observations at a later date the system was brought up to the same standards or a new series of standards was set up.

The method of analysis for lithium was carried out in the same manner, the wave length of the line observed being 6709 Å. Curves of time *vs.* concentration were drawn and the concentrations read quantitatively wherever possible. Since the amounts of lithium were very minute in all species except *Platanus occidentalis* the analysis is really quantitative for that species only.

The spectroscopic method used for calcium and magnesium has been described by several workers, namely, LEWIS (18), GERLACH (9), SMITH (25), and HOAG (14). The spectrograph (Bausch and Lomb Optical Co., Rochester, N. Y.) was equipped with a quartz optical system of the very best type and a photographic plate holder for taking 4" × 10" spectrograms.

Electrodes of graphite (Acheson Graphite Co., Niagara Falls, N. Y.) were employed. These were arced for 30 seconds before using in order to burn out any impurities, particularly the calcium and magnesium present.

There are many lines present in the arc spectrum of calcium and magnesium when these elements are present in the arc in fairly large amounts. The intensity of these lines depends upon the length of exposure, the power used by the arc, the characteristics of the instrument, the characteristics of the plate, and the time of development. However, as the concentration of these elements in the arc is decreased, the number of lines visible when other factors are held as constant as possible decreases until only the so called "raies ultimes" or ultimate lines (DEGRAMONT, 5) are present. These ultimate lines become less and less intense as the concentration of the substance in the arc decreases, the less sensitive lines fading out completely and the more sensitive lines remaining as the dilution is carried still further. Finally a point is reached at which the last of the lines fail to show in the spectrogram, and the presence of the element can no longer be detected at this or lower concentrations.

In order to make this method quantitative it is necessary to make spectrograms of known concentrations of the element in question, of the same order of magnitude as in the unknown, and to standardize the intensities of certain lines with respect to concentration. Two methods of procedure may be followed in this respect. In the first, the intensities of the spectral lines are measured as a function of the density of the line on the photographic plate

by a MOLL (19) recording microphotometer. This instrument provides a beam of light supplied by a bulb maintained at constant voltage which is focused on the line on the plate. The light transmitted by the line passes through a slit and falls upon a thermopile which is connected to a sensitive galvanometer, the mirror of which throws a light beam on a scale. The

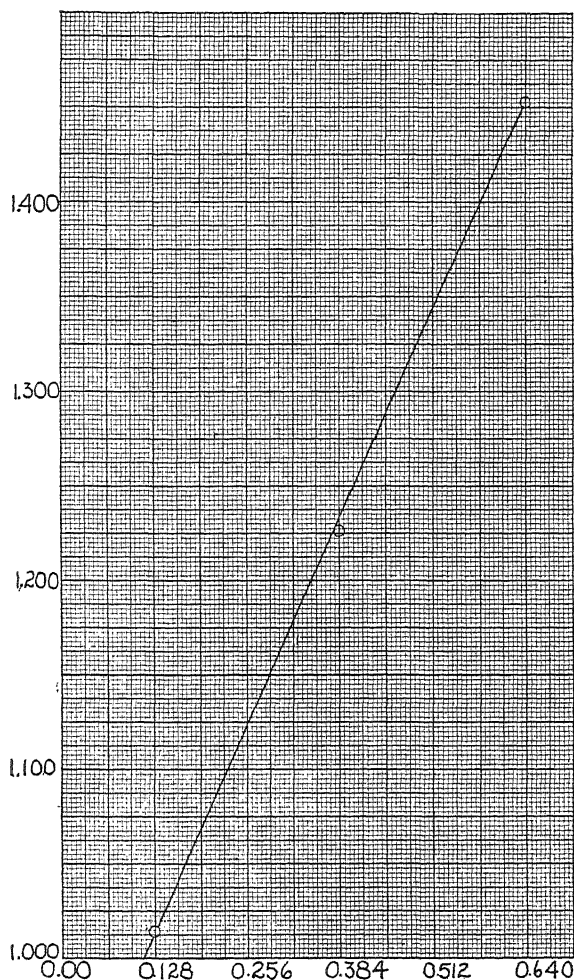


FIG. 2. Relation between concentration and density of magnesium line 2802 Å.

density of the line in question is taken by definition to be the  $\log_{10}$  of the ratio of the light incident upon the line to the light transmitted by the line. This ratio is obtained by taking a reading of the galvanometer with the light shining through the clear plate on either side of the line, which gives the maximum amount of light transmitted by the plate (M. T.). A reading is

taken with the light shut off to find the point of maximum blackness (M. B.). The plate is then moved into position so that the light shines directly through the line in question and the reading of the galvanometer is again taken (P.). The ratio is then expressed as  $\log_{10} \frac{M. T. - M. B.}{P. - M. B.}$ . Table II and figure 2 show the relation existing between concentration of element in the arc and density of the line produced.

TABLE II

RELATION BETWEEN CONCENTRATION AND DENSITY OF MAGNESIUM LINE 2802 Å.

CONCENTRATION OF MAGNESIUM	DENSITY
	$\log_{10} \frac{M. T. - M. B.}{P. - M. B.}$
<i>mg/cc</i>	
0.128	1.0133
0.384	1.2263
0.640	1.4523

Duplicate spectrograms of three different known concentrations are made on each plate along with duplicate exposures of seven samples of the material to be analyzed. The curve is drawn and the unknown concentrations are read from it.

The second method of determining the concentration of the solution in the arc consists of observing visually the concentrations at which certain lines are just visible and making comparison between the known concentrations and the unknowns. This is a good way to check the photometer method, but is not reliable enough to be employed as the sole method except when used by one long accustomed to such work. In this work all plates were read on the microphotometer and checked by the second method as well.

The substance to be analyzed was introduced into the arc in the following manner. A drop (volume 0.02 cc.) of the solution of the ash was placed on the hollowed out top of the lower electrode while it was still warm from the current which had burned out the impurities. The drop evaporated leaving a solid incrustation on the electrode always of the same quantity of material provided the concentration was kept constant. The arc was struck. The exposures were ten seconds in length, and the current was maintained at ten amperes from a 110 volt. D. C. line. Fresh electrodes were then introduced into the arc and other exposures made.

In order to rule out as far as possible errors resulting from plate differences and development, a series of known concentrations were run for each plate of unknowns and the concentrations of the unknowns read from the curve drawn for each individual plate.

Table III and figure 3 show the relation between concentration and density of line for calcium (wave length 3968 Å.).

TABLE III  
RELATION BETWEEN CONCENTRATION AND DENSITY OF CALCIUM LINE 3968 Å

CONCENTRATION OF CALCIUM	DENSITY
	$\text{Log}_{10} \frac{\text{M. T.} - \text{M. B.}}{\text{P.} - \text{M. B.}}$
<i>mg/cc</i>	
0.18	1.0133
0.54	1.1139
0.90	1.2180

The accuracy of the spectroscopic method of analysis for calcium, magnesium, and potassium was checked by ordinary analytical methods recommended by "Official Methods" (1930). The spectroscopic analyses for calcium and magnesium were found to vary by not more than 5 per cent. and for potassium by not more than 8 to 10 per cent.

Because the elements boron, copper, manganese, and strontium did not show up on the plates when the solutions of the ash were arced at their natural concentrations samples of material were ashed and then made up to much smaller volumes of solution of ash which concentrated the material to such an extent that qualitative analyses could be made for any of the rarer constituents.

The Gunning method modified to include the nitrogen of nitrates (21) gave the results on total nitrogen, and the nitrate nitrogen determination was made by a modification by FLEAR (8), for use with plant juice, of the phenol-disulphonic acid method of HARPER (11).

Identical methods were used for the treatment and analysis of the parenchyma sap which was collected during June of the following year. It was thought desirable to make these analyses on parenchyma sap in order to compare its composition with that of the phloem exudate from the identical trees.

## Results

Table IV shows the results of analyses of phloem exudate of seven trees of *F. americana*. It is noted that the pH value is considerably higher than the pH of the parenchyma sap as shown in table VII. Appreciable amounts of calcium, magnesium, and potassium are present in all cases while a trace of lithium is noted. The amount of reducing sugar is much smaller than sucrose except in no. 5, and the large quantities of sucrose present should be noted. Sufficient exudate was obtained in two cases only for the nitrogen determination. The amount of total nitrogen present is much greater than

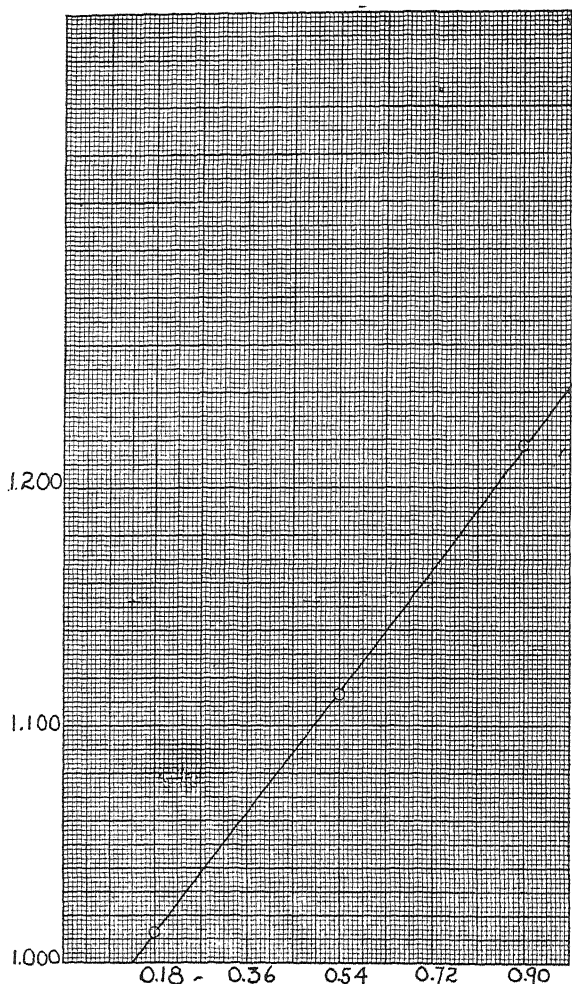


Fig. 3. Relation between concentration and density of calcium line 3968 Å.

the total nitrogen in the phloem exudate of any other species studied. Also there is an appreciable amount of nitrate nitrogen present.

Results in table V show that the phloem exudate of the two species included in it have rather high pH values, appreciable amounts of calcium, magnesium, and potassium, and a trace of lithium. The reducing sugars are consistently low, but the sucrose is exceptionally high, particularly in *Robinia pseudo-acacia*. Nitrate nitrogen also is present.

It is evident from table VI that the phloem exudate of *Platanus occidentalis* has larger amounts of lithium than any of the other species studied, and that the amount of reducing sugar is greatly increased in absolute

TABLE IV  
PHLOEM EXUDATE ANALYSIS OF *Fraxinus americana*

TREE NO.	DATE							
	SEPT. 2	SEPT. 20	SEPT. 10	AUG. 1	AUG. 1	AUG. 5	AUG. 15	AUG. 15
	1	2	3	4	5	6	7	Av.
pH	7.15	7.07	7.30	7.05	7.20	7.49	7.25	7.22
Ca mg/cc	1.18	1.09	1.04	0.55	0.76	0.86	0.34	0.83
Mg mg/cc	0.53	0.21	0.44	0.18	0.32	0.36	0.24	0.33
K mg/cc	0.99	0.45	0.64	1.20	1.02	0.58	1.12	0.86
Li mg/cc	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
REDUCING								
SUGARS	11.12	Trace	0.84	0.22	30.18	2.64	1.98	7.83
mg/cc								
SUCROSE								
mg/cc	72.64	70.54	77.40	102.18	65.46	93.71	86.45	81.20
TOTAL								
SUGARS	83.76	70.55	78.24	102.4	98.64	96.35	88.43	88.34
mg/cc								
NITRATE								
NITROGEN		0.11	0.095					0.103
mg/cc								
TOTAL								
NITROGEN		2.45	3.64					3.05
mg/cc								

TABLE V  
PHLOEM EXUDATE ANALYSIS

TREE NO.	<i>Fraxinus pennsylvanica</i> var. <i>lanceolata</i>		<i>Robinia pseudo-acacia</i>			
	DATE		DATE			
	SEPT. 1	AUG. 20	SEPT. 1	SEPT. 15	AUG. 5	AUG. 5
	1	2	1	2	3	4
pH	7.58	7.20	7.4	7.36	7.23	7.25
Ca mg/cc	1.04	1.33	0.90	0.44	0.72	0.72
Mg mg/cc	0.61	0.21	0.38	0.15	0.39	0.38
K mg/cc	0.37	0.58	1.14	1.21	0.90	0.95
Li mg/cc	Trace	Trace	Trace	Trace	Trace	Trace
REDUCING						
SUGARS						
mg/cc	3.41	3.81	2.08	4.78		0.51
SUCROSE						
mg/cc	107.99	118.09	177.82	147.94		199.94
TOTAL						
SUGARS						
mg/cc	111.4	121.9	179.9	152.72		200.45
NITRATE						
NITROGEN						
mg/cc	0.12					0.135
TOTAL						
NITROGEN						
mg/cc	0.49					0.56

TABLE VI  
PHLOEM EXUDATE ANALYSIS

TREE NO.	<i>Platanus occidentalis</i>				<i>Cucurbita maxima</i>	
	DATE				DATE	
	SEPT. 20	AUG. 30	AUG. 30	SEPT. 10	FEB.	FEB.
	1	2	3	4	1	2
pH	7.35	7.31	7.28	7.31	8.1	8.0
Ca mg/cc	1.26	1.26	0.76	0.84	0.18	0.07
Mg mg/cc	0.66	0.24	0.28	0.19	0.06	0.10
K mg/cc	0.96	0.80	0.90	0.90	0.24	0.20
Li mg/cc	0.08	0.04	0.08	0.158	None	None
REDUCING SUGARS mg/cc	86.4	67.3		56.5	4.83	2.07
SUCROSE mg/cc	1.90	40.3		48.4	2.83	3.33
TOTAL SUGARS mg/cc	88.30	107.6		104.9	7.66	5.4
NITRATE NITROGEN mg/cc	0.203					
TOTAL NITROGEN mg/cc	0.28					

amounts and also in ratio to the total sugars. This increase in reducing sugar may be due in part to the change of sucrose to reducing sugar during the interval between collection of the exudate and analysis. The use of toluene as a preservative, while preventing the growth of organisms in the exudate, would not hinder the action of any enzymes which might cause inversion of the sucrose. The high pH of *Cucurbita maxima*, and the lower concentrations of all its solutes are noted. Insufficient samples were obtained to allow for nitrogen determinations.

In order to make comparisons between phloem exudate and parenchyma sap from the same plants, similar analyses were carried out for the samples of parenchyma sap collected as described previously. Results of these analyses are included in table VII and show low pH values of parenchyma sap for all species studied with respect to the pH of the phloem exudate. In general the concentrations of calcium, magnesium, potassium, lithium, and total and nitrate nitrogen are about the same in parenchyma sap as in phloem exudate. Whether or not leaf sap and phloem exudate would have been more similar or more divergent if taken at the same time cannot be determined from these data. The amounts of reducing sugars are in excess of the sucrose in all cases while the total sugar concentration is much less than that of the phloem exudate.



TABLE VII  
PARENCHYMA SAP ANALYSIS

TREE NO.	<i>Fraxinus americana</i>	<i>Fraxinus pennsylvanica</i> var. <i>lanceolata</i>	<i>Robinia pseudo-acacia</i>	<i>Platanus occidentalis</i>	<i>Cucurbita maxima</i>		
	DATE						
	JUNE 5	JUNE 5	JUNE 5	JUNE 5	JUNE 5	JUNE 5	
	2	3	1	2	1	2	
pH	5.63	5.70	5.44	5.80	6.82	4.93	6.11
Ca mg/cc	0.72	1.08	1.08	0.72	0.90	1.19	0.54
Mg mg/cc	0.38	0.64	0.58	0.32	0.32	0.69	0.12
K mg/cc	0.86	1.03	0.93	0.64	1.07	0.74	0.69
Li mg/cc	Trace	Trace	Trace	Trace	Trace	Trace	Trace
REDUCING SUGARS mg/cc	13.66	12.07	12.01	6.36	3.1	10.26	3.50
SUCROSE mg/cc	2.20	1.87	7.18	4.06	1.68	5.45	1.33
TOTAL SUGARS mg/cc	15.86	13.94	19.19	10.42	4.78	15.71	4.83
NITRATE NITROGEN mg/cc	0.045	0.045	0.087	0.077	0.104	0.098	0.075
TOTAL NITROGEN mg/cc	0.070	0.056	0.28	0.112	0.14	0.14	0.84

Qualitative spectroscopic analyses of concentrated solutions of the ash of both phloem exudate and parenchyma sap indicate the presence of barium, boron, copper, manganese, and strontium in varying undetermined amounts in all cases.

### Discussion

It has been generally accepted for some time that organic materials are transported through the phloem tissues of plants. The presence of large amounts of organic materials such as sugars, which may reach values as high as 20 per cent. of the exudate, and nitrogenous compounds in the phloem exudate of several species tends to support this idea of transport of organic materials through the phloem. There has not been the same unanimity of agreement with respect to the transport of inorganic materials. The majority of investigators claim that inorganic materials are carried upward primarily through the xylem. CURTIS (4) however has presented irrefutable data that inorganic as well as organic materials may be transported through the phloem tissues. The presence in the phloem exudate of such inorganic constituents as calcium, magnesium, potassium, and nitrate nitrogen in amounts appreciably greater than in the parenchyma sap, as well as the presence of small quantities of barium, boron, copper, manganese, and

strontium lends support to the idea of transport of inorganic materials through the phloem tissues. The absence of an element from the phloem would indicate that it was not transported there while the presence of an element in the phloem exudate would offer presumptive but not conclusive evidence of its transport through those tissues.

An interesting observation was made in connection with the flow of the exudate. Collection was begun about August 1st and the flow was good until the first week in October just before the leaves began to change color and fall. The following spring no flow from the phloem tissues could be obtained until about June 15 at which time it was very meager. By July 15 the flow was approximately equal in volume to the flow in late summer of the previous year. The fact that the flow was very slight during the spring and early part of the summer when the cambium is most active lends support to the idea that the exudate was almost wholly limited to the phloem.

It was noted that the total sugar concentration of *Fraxinus americana* decreased as the samples were taken later in August and in September.

Mention has been made of the effect of the proximity of a second cut on the flow from a previous cut.

### Summary

1. The pH value of phloem exudate was found to range between 7.05 and 8.10 while that of parenchyma sap ranged between 4.93 and 6.82.

2. Calcium, magnesium, potassium, and lithium were found to be present in the phloem exudate sometime in amounts greater than in the parenchyma sap.

3. The amount of reducing sugar in the phloem exudate is much less than the amount of sucrose except in the case of *Platanus occidentalis*, and the reducing sugar here may have resulted from hydrolysis of sucrose after collection.

4. The total sugar concentration of phloem exudate is much greater than that of the parenchyma sap and may reach 20 per cent. of the exudate.

5. In parenchyma sap the amount of reducing sugar present exceeds the amount of sucrose.

6. Total nitrogen, and nitrate nitrogen concentrations of phloem exudate exceed those of parenchyma sap.

7. Barium, boron, copper, manganese, and strontium are present in both the phloem exudate and parenchyma sap.

8. The presence of appreciable amounts of inorganic constituents in the phloem exudate is presumptive evidence that inorganic as well as organic materials are transported through the phloem.

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CORNELL UNIVERSITY

ITHACA, NEW YORK

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# CARBOHYDRATE METABOLISM OF *VITIS VINIFERA*: HEMICELLULOSE

A. J. WINKLER AND W. O. WILLIAMS

## Literature

A review of plant science textbooks reveals the general opinion that hemicellulose often functions as a reserve food. This viewpoint has probably arisen from consideration of the early findings of SACHS (36), REISS (34), and SCHULZE (37) that hemicellulose appears to be a major food in certain seeds.

The more recent studies on the composition of hemicellulose have led to other theories as to its physiological function besides those of reserve food and structural reinforcement. O'DWYER (31) has suggested that since hemicellulose is closely related to pectins it serves as an intermediary form in a synthesis of lignin from pectic compounds. The support for this theory is founded on the supposed decrease of pectin during the period of tissue maturation and on the apparently successful attempts to convert pectins to hemicelluloses by mild alkaline hydrolysis (5), by hydrolysis with water under pressure (21), and by mild oxidation (28). The last reaction is presumably analogous to conversion *in situ* in the plant. Although it has been possible to demonstrate the transformation of pectin into hemicellulose, there is no support for the further transformation of hemicellulose into lignin (32). Since the structure of lignin has not been established, the theory of the transformation of hemicellulose into lignin must be considered as speculative. KERR and BAILEY (17) in their microchemical investigations of the cell wall, moreover, found no reason to believe that pectins disappear with cell maturation. A number of investigators have isolated a small amount of pectin substances from mature wood. The apparent percentage decrease in pectin accompanying maturation appears to be the result of an increasing proportion of other constituents. Difficulties of extraction engendered by mechanical protection or perhaps by a physico-chemical union afforded by the later deposits may possibly have decreased the analytical yield of pectin. Hence positive evidence that the absolute amount of pectin decreases is lacking.

Large variations of any of the constituents, notably that of the labile carbohydrate reserves, cause an inverse change in the percentage of other components. This is illustrated by the hemicellulose content of the fruit of the apple (13, 24), the peach (27), and the pear (22). In each case with maturation a decreasing hemicellulose content was found on a percentage basis which led to the opinion that hemicellulose is utilized as a reserve food in the development of the fruit. Where the hemicellulose content has been expressed on a single fruit basis, however, there is a steady increase in the

absolute amount of hemicellulose in the fruit during growth and ripening, as WIDDOWSON (42) has demonstrated for the apple, and SMITH (38) for the Kieffer pear. In other words, hemicellulose deposition has only slowed down in respect to the rate of deposition of other substances in the fruit. Working with flowers and young fruits of the apple, HOWLETT'S (14) data show no such rapid reversible changes of hemicellulose to sugars as MURNEEK (24) found. WIDDOWSON'S data seem to show a slight decrease in the absolute amount of hemicellulose as the fruit becomes overripe, but this might be the result of tissue disintegration which in all probability has no connection with the utilization of hemicellulose as a reserve. BUSTON (4), studying the pods of ripening peas and beans, found a decrease in the hemicellulose content on a percentage basis but the data for hemicellulose in grams per hundred pods showed a steady increase.

Sufficient care has not always been taken to formulate an adequate conception of a reserve food. GARDNER, BRADFORD, and HOOKER (12) state, "The seasonal variation in the carbohydrate of plants gives evidence of storage." It seems necessary to make the further limitation that fluctuations which do not clearly result from conversion to other forms (such as starch to sugar in early winter) should correlate with conditions of vegetative activity which lead to utilization or storage of reserves. A large percentage utilization under extreme carbohydrate depletion should also be observable. In this latter connection the variability in composition of the hemicellulose extract may raise the question as to whether or not we are dealing with the extraction of a homogeneous fraction or with the simultaneous extraction of several fractions of hemicellulose. Recent studies of hemicellulose by O'DWYER (30), NORRIS and PREECE (29), and EHRLICH (11) have indicated its varying composition—the hydrolysis products yielding hexose and pentose sugars together with a quantity of uronic acid. One such constituent or group of constituents may serve a structural function; the other, perhaps a minor fraction, may serve as a reserve food. If this were the case the utilization of a large proportion of the hemicellulose present would not be observable.

CZAPEK (7) and KOSTYTSCHIEW (18) and others have emphasized the importance of particular hemicellulose constituents as reserves, indicating a less prevalent belief that only certain fractions of the hemicellulose group may function primarily as reserves.

Still another type of finding that has led to an apparently erroneous conclusion is illustrated by the data of NELSON (25) and of LEUKEL (20) which show small decreases in hemicellulose in alfalfa roots that had undergone repeated top cutting treatments, and of NIGHTINGALE (26) which show a significant percentage decrease of hemicellulose in tomato stems grown in darkness for an extended period. These decreases most likely represent the

net effect of a lessened hemicellulose deposit in the newly formed tissue on the percentage of the hemicellulose in the entire plant part utilized. This is confirmed by ALBERT's (1) data on alfalfa which show that new top growth produced in the dark room had a hemicellulose percentage much lower than normal. The difference would have been larger had the hemicellulose content of the normal material been expressed as percentage on a carbohydrate-(starch and sugar) free basis. The evidence seems to indicate that a larger or smaller quantity of hemicellulose may be laid down according to the nutritional conditions within the plant.

A number of investigations show no particularly significant variations in the hemicellulose content. Only a few of these need be mentioned, chiefly to indicate some of the plants that have been studied. DENNY (10) seems to have found no consistent variation in the hemicellulose content of leaves of tobacco, sunflower, cotton, grape, hawthorn, peach, bean, and lilac, using twin-leaf samples collected at night and in the morning. On the average his results indicate a slight gain. JONES and BRADLEE (16) found very small seasonal variations in the maple tree. BENNETT (2) in a seasonal study of the apricot found no reason to believe that hemicellulose functioned as a reserve. WILLIAMS (43) working with the dewberry found that the acid hydrolyzable material showed minor seasonal changes. L. D. Davis (unpublished data), working with bearing and non-bearing trees of the sugar prune, found no significant seasonal variation in hemicellulose although large fluctuations occurred in the starch content (8) of the same material.

MURNEEK (24), LEONARD (19), and CLEMENTS (6) support the theory that hemicellulose serves as a reserve food in the plant. In their investigations, the observed variations of the hemicellulose content were irregular, and furthermore, are not correlated with the vegetative condition of the plant. Their variations certainly do not point to hemicellulose as a reserve, but may have arisen largely from sampling and analytical errors. Arbitrary chemical determinations of hemicellulose may show comparatively large deviations.

Several investigators have employed treatments leading to carbohydrate utilization and have studied the effect on the hemicellulose content. DELEANO (9) divided carefully selected leaves of *Vitis vinifera* along the midrib. One portion served as a check and the other was placed in a light-excluding respiration chamber for varying periods of time from 22 to 288 hours. Under these conditions of decreasing carbohydrate reserves, the percentage of hemicellulose seemed to increase slightly. This increase might be ascribed to continued hemicellulose deposition in the leaf, but it more probably was caused by the effect of the decrease of other constituents on the percentage of hemicellulose. A slight increase of total nitrogen was also shown. If the hemicellulose data are corrected, using the nitrogen increase as a basis, the resultant figures for hemicellulose are remarkably constant. Although

BUSTON (4) found a decrease in the hemicellulose content in grape leaves held in darkness, however, his findings are limited. PROEBSTING (33) in experiments on the apple found that continued defoliation did not decrease the hemicellulose content. The work of ALBERT (1), in which he endeavored to exhaust the reserve from alfalfa roots by growth under conditions of light exclusion, resulted in a consistent increase in the percentage by weight of hemicellulose, doubtless an effect of the carbohydrate utilization on the percentage composition of the residue. It is apparent that hemicellulose was not utilized to any appreciable extent as a reserve under these conditions.

### Experimentation

The analytical data, presented in table I, were obtained from representative cross-sections of grape stems (*Vitis vinifera* var. Carignane). The labeling of the sections selected, as indicated in table I, is self-explanatory, except that the term shoot is used to indicate the herbaceous growth of the current season as opposed to the designation cane, used to indicate the mature stems of the preceding season. The data on the annual hemicellulose level were obtained by analysis of composite samples of each of the various sections, obtained by the removal of ten vines at each sampling, at intervals varying from a few days up to one month during the course of the year period. The same sampling procedure was utilized in the severe defoliation treatment, except that only six vines were removed for the composite sample. The defoliation treatment consisted of leaf removal at intervals sufficiently short that the leaves never reached a diameter of more than 2 inches. The defoliation was repeated until the vines were unquestionably in a dying condition. Adjacent vines receiving the same treatment were dead at the time of sampling. The composite samples were dried rapidly in a strong air blast at 55° to 60° C.

The sugars were removed by extraction for 6 hours with 95 per cent. ethyl alcohol. The starch was liquefied by buffered taka-diestase (previously precipitated from water solution by alcohol to remove most of the blank). The residue from the starch extraction was hydrolyzed with 2 per cent. HCl (by weight) for three hours in an enclosed steam bath. The resulting sugars were determined by the MUNSON-WALKER-SHAFFER-HARTMAN method.

It is realized that this analytical procedure includes other polyuronides which are present (chiefly pectic substances) but there is no reason to believe that this affects the conclusions drawn. The data represent averages of the results of duplicate analyses of each sample. All results are expressed in terms of dextrose as percentage of the dry carbohydrate-free residue (starch and sugars) following the example of MASON and MASKELL (23). DENNY (10) found that this procedure gives results compatible with those calculated on the unit organ basis. Large variations in the stored starch have intro-



TABLE I  
HEMICELLULOSE CONTENT OF *Vitis vinifera* VINE SECTIONS. PERCENTAGE ON A CARBOHYDRATE-FREE DRY WEIGHT BASIS

DATE	NOV. 6	DEC. 6	JAN. 8	FEB. 10	MAR. 10	MAR. 25	APR. 1	APR. 20	MAY 5	MAY 13	MAY 26	JUNE 7	JULY 6	AUG. 8	SEPT. 6	OCT. 6	NOV. 6	AVER- AGE	REPEAT- EDLY DEFOLI- ATED VINES
Rootlets .....	10.4	11.0	10.3	10.9	11.0	11.2	10.5	11.2	10.9	10.5	10.8	11.6	10.1	10.5	11.0	11.2	11.0	10.8	14.7
1 year root wood ..	13.1	13.4	13.5	13.1	14.1	14.3	14.1	14.3	14.1	13.5	14.8	15.4	14.3	14.3	14.5	14.7	14.5	14.1	7.5
1 year root bark ..	8.0	8.1	7.7	8.7	8.8	7.1	7.7	7.8	7.7	7.7	8.3	8.4	7.6	7.6	7.8	7.6	7.4	7.9	15.6
2 year root wood ..	15.6	15.7	16.0	15.6	15.4	15.7	16.3	15.7	16.3	17.1	16.8	16.9	17.1	17.0	15.5	16.4	16.0	16.2	7.9
3 year+trunk wood	10.7	9.9	10.0	10.0	10.1	9.6	9.9	8.4	8.5	10.2	9.8	8.6	8.8	10.3	9.3	8.9	9.1	19.7	20.0
2 year trunk wood	19.2	19.4	20.2	22.4	21.7	21.3	22.6	20.6	20.6	21.6	20.0	20.1	19.8	19.7	19.1	19.8	19.3	21.2	20.8
1 year trunk wood	19.9	19.5	19.8	19.9	19.6	19.9	19.4	20.9	20.9	20.7	20.2	20.8	20.4	20.5	20.9	20.5	21.0	20.2	20.5
Trunk bark .....	10.8	11.8	12.1	11.7	11.8	10.4	11.4	9.8	11.4	10.9	12.5	10.3	11.9	11.1	11.7	12.4	12.1	11.4	12.4
Canes basal wood	21.8	21.0	20.6	20.2	20.7	20.4	21.3	21.1	21.1	22.1	21.6	21.7	21.5	21.7	21.1	20.7	20.0	21.1	20.2
Canes basal bark	14.1	13.8	13.5	14.4	15.1	15.0	13.6	14.0	12.7	13.5	12.7	12.8	13.5	12.5	13.8	14.1	13.8	13.7	14.3
Canes middle wood	19.3	20.0	19.3	19.5	19.2	19.7	19.8	19.8	20.5	19.3	19.4	19.7	20.3	—	—	—	13.8	14.1	17.3
Canes middle bark	14.2	14.4	13.6	15.3	15.6	16.1	15.7	13.6	13.3	13.3	12.4	12.2	14.5	—	—	—	13.8	14.1	17.3
Shoot basal wood								7.0*	11.9*	16.1	17.9	16.2	17.0	17.6	18.4	17.7	17.8	17.3	12.9
Shoot basal bark ..								11.5	12.4	13.0	12.3	12.5	12.0	13.4	14.0	13.7	13.9	17.6	17.6
Shoot middle wood										16.2	18.0	17.2	17.6	18.4	17.6	17.8	18.3	13.3	13.3
Shoot middle bark										12.9	12.3	12.6	11.8	13.5	14.6	14.8	14.1	6.5	6.5
Mature leaves .....	6.4							6.7	7.1	6.8	6.6	7.0	6.6	6.6	5.7	5.9	—	—	—

\* Not included in the average.

duced apparent but erroneous inverse fluctuations in the data on the hemicellulose content.

### Discussion

The most striking feature of the data (table I) is the lack of significant variation between the samples of each series except that for the base of the shoots. In the first sample of this series, taken when shoots were very succulent, the hemicellulose content was less than half that of the usual content (approximately 7 per cent. against the average of later values of 17.3 per cent.) after the tissues had become more mature. At the second sampling, when the base of the shoot was becoming woody, there was still a significantly low hemicellulose content in this section of the vine (12 per cent.). It is interesting to note that these first samples present an inverse curve of the reducing sugar content which decreases rapidly between May 5 and May 26. Starch deposition was relatively very small until approximately a month after the hemicellulose had attained its normal magnitude. At this time the base of the shoot was no longer succulent.

The appearance of a normal hemicellulose content during the period of rapid growth and before starch storage actively begins, points to its function as a structural material. This theory is supported by the lack of significant fluctuation in the hemicellulose content in the other sections. The starch content, which is the principal reserve in this case, fluctuated widely in each part of the vine represented by the samples analyzed. In certain cases (bark samples of canes and trunk) starch was absent during the period of the summer minimum. The non-utilization of hemicellulose in periods when the starch is deficient in certain areas of the vine or at the low summer minimum seems to show that it does not normally serve as a reserve food in the vine.

Although the above results indicate that hemicellulose does not function normally as a reserve food in the vine, it seemed desirable to determine whether or not it *could be utilized* as a reserve of "last resort" as has been proposed by NIGHTINGALE (26). In the vines dying because of repeated defoliation treatments, the hemicellulose was found to be present in normal amounts. It is apparent (see table I) that hemicellulose was not utilized as a food reserve even under these conditions of abnormal depletion of carbohydrate reserves. There always remained a detectable amount of starch (with iodine test) in the woody stems. Perhaps this residue was unavailable or was being hydrolyzed very slowly to soluble carbohydrates.

The data for the average hemicellulose values of the various vine cross-sections in table I appear to support the theory of the structural function of hemicellulose. There is a correlation with general observations on comparative thickness of all walls—structural rigidity—of the various tissues with their respective hemicellulose contents. The hemicellulose content was greatest in the xylem of the woody stem, successively decreasing in the

root-wood, bark, and leaves. A number of investigators, including THOMAS (40), PROEBSTING (33), and SMYTH (39), have noted the difference in hemicellulose content between the wood and the bark of the stem. One year MURNEEK (24) found less hemicellulose in leaves than in wood but not consistently less in the following year. These differences are presumably caused by the greater amount of hemicellulose in the walls of xylem cells than in those of phloem and cortex cells. The decreased ratio of cortex to xylem in older roots correlates with the increasing hemicellulose content (table I). BENNETT'S (2) data show distinctly less hemicellulose in lateral roots than in trunk wood for the apricot.

The mature wood had slightly more hemicellulose than the young shoots. This is an agreement with the results of TOTTINGHAM, ROBERTS, and LEPKOVSKY (41) who found less hemicellulose in the new spur growth as compared with that of the old spurs or base wood. Neither of these findings seems to agree with the conclusions of BRADBURY AND ROBERTS (3) that acid hydrolysis attacks the young xylem, phloem, and cambium cells rather than the "reserves" occurring as wall thickenings. Probably the increased hemicellulose content of older wood is caused by further hemicellulose deposition. It is reasonable, however, to assume that nutritive or other conditions might reverse this result by influencing the amount of the hemicellulose deposit which is laid down or at least might affect its percentage ratio.

### Conclusions

A critical review of the data presented in the literature reveals lack of definite evidence to support the assumption that hemicellulose functions as a reserve food in the vegetative part of plants. On the contrary, a preponderance of evidence indicates that it does not function as a reserve. Definite evidence of its utilization in the seeds of a few plants seems to have been obtained but it appears wise to avoid the reasoning that if hemicellulose is a reserve food in the seed, it will necessarily perform this same function in the plant.

The data of this investigation obtained from *Vitis vinifera* stems fail to show any indication of the metabolic utilization of hemicellulose and support the theory that hemicellulose does not function as a reserve material in the vine even as a reserve of "last resort." The data further indicate that hemicellulose probably functions as a structural material.

UNIVERSITY OF CALIFORNIA  
DAVIS, CALIFORNIA

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# ACCESSORY SALTS IN THE NUTRITION OF EXCISED TOMATO ROOTS

PHILIP R. WHITE

(WITH FOUR FIGURES)

## Introduction

In a recently reported study of the effect of vitamin B<sub>1</sub> on the growth of isolated tomato roots (9), evidence was obtained which showed that the "accessory salts" regularly included in the medium employed in earlier work (7, 8) constituted a growth-limiting factor. Vitamin B<sub>1</sub> proved to be beneficial only in the presence of these salts. That result has since been repeatedly verified. The salt mixture used was modeled after that of TRELEASE and TRELEASE (3). It was thought that the ions of its 12 salts might take the place of unidentified but important ash constituents contained in the yeast extract which it was desired to replace. No effort was made to determine the relative importance of the constituents.

With the establishment, however, of a synthetic nutrient capable of maintaining continuous growth of isolated roots, at a fairly satisfactory level, a quantitative examination of all its constituents appeared to be an essential step in attempting further improvement of this nutrient (9). The accessory salt mixture was the only portion of the nutrient which had not been subjected to at least a preliminary examination of this sort (4, 7, 8, 9). The observations of ROBBINS, WHITE, McCLARY, and BARTLEY (2) also suggested that the ash constituents might be worthy of closer examination.

The present paper records the results of a quantitative study of the effects of each of the components of this accessory salt mixture. The materials and general methods employed were the same as those reported in earlier papers (7). The roots had been maintained for 200 or more passages under standard conditions (6) before being subjected to experimental treatments.

## Experimental results

The accessory salt solution under consideration contained in each liter of complete nutrient the following salts in the amounts shown below:

Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .....	1.07 mg.	KI .....	0.15 mg.
NaCl .....	0.56 "	As <sub>2</sub> O <sub>3</sub> .....	0.15 "
MnSO <sub>4</sub> .....	0.44 "	NiCl <sub>2</sub> .....	0.04 "
H <sub>3</sub> BO <sub>3</sub> .....	0.32 "	CoCl <sub>2</sub> .....	0.04 "
ZnSO <sub>4</sub> .....	0.30 "	LiCl .....	0.03 "
Na <sub>2</sub> SiO <sub>3</sub> .....	0.27 "	CuSO <sub>4</sub> .....	0.01 "
Total 3.38 mg.			

Since it had previously been found that concentrations of the standard salts optimal for tomato roots were about one-tenth those commonly recommended

for entire plants (4), the concentrations of the accessory salts were likewise adjusted to about one-tenth those recommended by TRELEASE and TRELEASE (3). The optimal concentration of the entire mixture was then determined experimentally. A series of solutions was prepared containing sugar, standard salts, amino acids, and vitamin B<sub>1</sub> at the standard concentrations (7, 8, 9) and the accessory salts at 1, 3, 10, 30, and 50 times the concentrations tabulated above. Twenty cultures were grown at each concentration. Cultures were carried through three consecutive passages, each of one week duration. They were measured daily, the measurements averaged to obtain a weekly index and this recorded as percentage of the control grown in a nutrient containing yeast extract. A "negative control" was also maintained, in which neither yeast extract nor its equivalent (amino acids, vitamin B<sub>1</sub>, and accessory salts) was used. The results are shown in figure 1. In the first

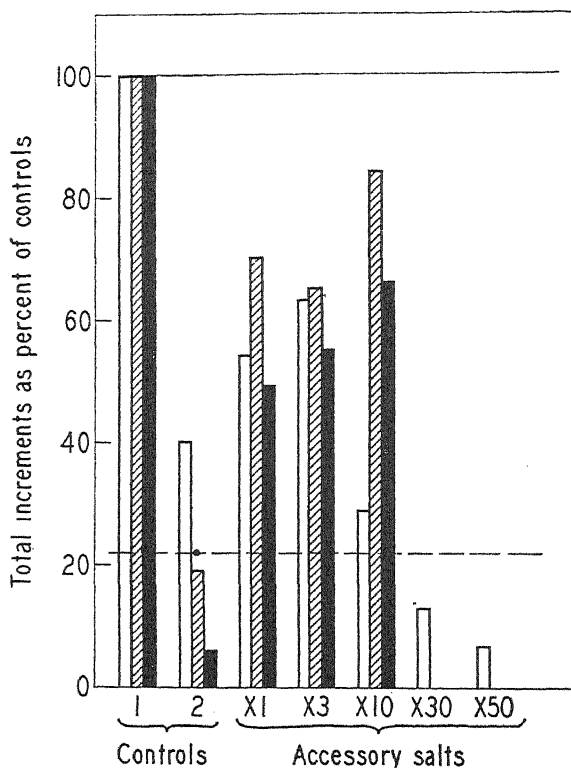


FIG. 1. Growth rates of excised tomato roots in a nutrient containing sugar, standard salts, amino acids, and vitamin B<sub>1</sub>, to which various concentrations of an accessory salt mixture were added. Control 1 contained yeast extract in place of amino acids and vitamin. Control 2 was without organic material other than sugar. Open columns represent the first, hachured the second, and blocked-in columns the third passages. The transverse dotted line is at the level of the average growth rate (3 passages) without accessory material. Any growth rate below this level indicates a depressant effect.



passage growth at concentrations 10, 30, and 50 times the standard was less than in the negative control, that is, these solutions had a depressant effect. The last two concentrations were, therefore, abandoned. Growth was best at a concentration 3 times the standard in the first passage, but there was a gradual recovery at 10 times the standard so that in the second and third passages this nutrient gave superior results. It thus appears that tomato roots may become adapted to growth in salt concentrations higher than those which are at first optimal. For subsequent studies a concentration between these two values, 5 times the original standard, was chosen. Such a nutrient contained 17 mg. of accessory salts per liter.

The importance of each salt was then studied in an "all or none" series. Sixteen nutrients were prepared. Each, with the exception of the negative control, contained the standard salts, sugar, amino acids, and vitamin B<sub>1</sub>. Four controls were used, one containing yeast extract, one without accessory material but with standard salts and sugar, one containing all 12 of the accessory salts, and one from which all these salts were omitted. In each of the remaining solutions one of the 12 accessory salts was omitted. Twenty cultures were prepared in each nutrient and were maintained through 3 passages. The results are shown in table I.

Of the 12 salts studied, only 2, aluminum sulphate and potassium iodide,

TABLE I

GROWTH INDICES OF TOMATO ROOTS CULTIVATED IN NUTRIENTS EACH LACKING A SINGLE ONE OF THE ACCESSORY SALTS, EXPRESSED AS PERCENTAGE OF THE GROWTH IN A CONTROL NUTRIENT CONTAINING YEAST EXTRACT

SOLUTION	PASSAGE			AVERAGE
	1	2	3	
With yeast ..... 1	100	100	100	100
Without yeast ..... 2	34	43	17	28
All accessory salts omitted ..... 3	68	50	49	56
All accessory salts included ..... 4	100	79	95	91
Minus Al .....	108	106	110	108
“ Li .....	88	93	110	97
“ Zn .....	102	81	95	93
“ Mn .....	92	74	102	89
“ Ni .....	98	65	97	87
“ Cu .....	91	84	85	87
“ Si .....	82	85	96	87
“ Na .....	97	86	81	87
“ Co .....	90	79	85	85
“ As .....	102	74	64	80
“ B .....	73	74	80	76
“ I .....	64	56	60	64

gave sufficiently clear-cut results to be of certain significance. Aluminum sulphate (1 p.p.m.) was clearly detrimental. Potassium iodide (0.15 p.p.m.) was equally clearly beneficial. All other salts were of doubtful value. In view of the uncertainty of these results, an arbitrarily chosen combination was selected for further study. For this, 6 salts only were chosen: potassium iodide because the preliminary study had suggested its importance; manganese and zinc sulphates and boric acid because, in spite of their apparent unimportance in this first study, the literature of plant nutrition contains so many rather clear-cut demonstrations of their desirability (the first two are likewise frequently found as impurities in salts of other metals and may have been supplied in sufficient amounts in the 11 salts present in the "minus Mn" and "minus Zn" series); sodium silicate because MAST and PACE (1) have suggested its importance for certain organic catalyses; and cobalt chloride because of the recent interest in the possible rôle of cobalt in animal and plant nutrition. WINKLER years ago (10) pointed out the possible growth-stimulating effects of traces of cobalt. Since it was desired to test 4 concentrations of each salt and since the maximum number of cultures which could be maintained at one time under uniform conditions in the laboratory available was 320, no more than 4 salts could be compared simultaneously. Salts of iodine, boron, manganese, and silicon were studied first. Nutrients were made up containing 5 of the 6 salts chosen, at standard concentrations, while the sixth salt was introduced at 0.0, 0.1, 1.0, and 10.0 times the concentrations employed in the earlier series. The experiment was conducted as before.

The results, shown in figure 2, indicate clearly that iodine was essential

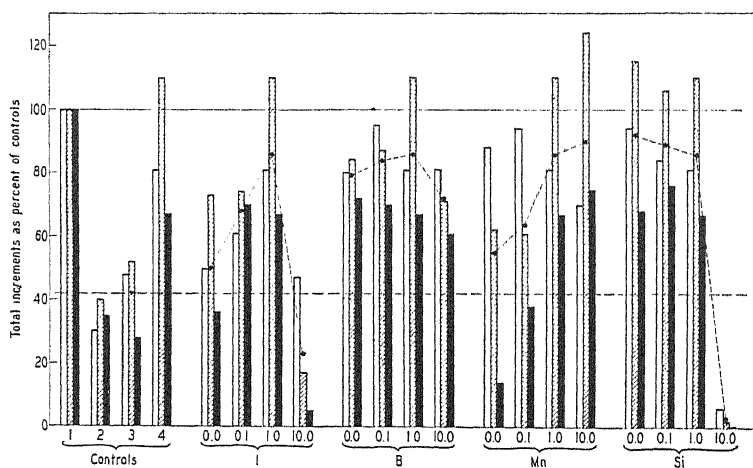


Fig. 2. Legend as for fig. 1. Control 3 is with amino acids and vitamin B, but without accessory salts. Control 4 is the same as 3 but with I, B, Mn, Si, Zn, and Co added (see text). The transverse dotted line is placed at the level of the mean growth rate for control 3, without accessory salts. Solid dots are in each case placed at the average (3-week) growth rate for the nutrient indicated.

for satisfactory growth of isolated tomato roots. A concentration of about 0.75 p.p.m. of KI was about optimal. Manganese was likewise highly desirable, with an optimal concentration at about 4.40 p.p.m. of  $\text{MnSO}_4$ . Enough Mn seems to have been present in the "minus Mn" nutrient for fairly satisfactory growth. Boron was possibly beneficial with an optimum at about 1.60 p.p.m. of  $\text{H}_3\text{BO}_3$ . Some boron was, of course, supplied to the cultures by solution of the glass (Pyrex) in which they were grown. A clear-cut deficiency was thus not to be expected in this case. Silicon proved to be markedly detrimental, the growth rates showing a regular diminution with increasing concentration of  $\text{Na}_2\text{SiO}_3$ . In two solutions, that with increased Mn concentration and that from which silicon was omitted, the growth indices were greater than when all 6 salts were present at the concentrations used before, and were superior to any results obtained in earlier nutrients containing all 12 salts.

Using these results as a basis, a series was then prepared in which Si was omitted, the concentrations of I and B were maintained at the basic values, the concentration of Mn was increased by 10, and the concentrations of Zn and Co were varied as was previously done with the other ions. The results, shown in figure 3, indicate that zinc was probably beneficial, with an optimum at about

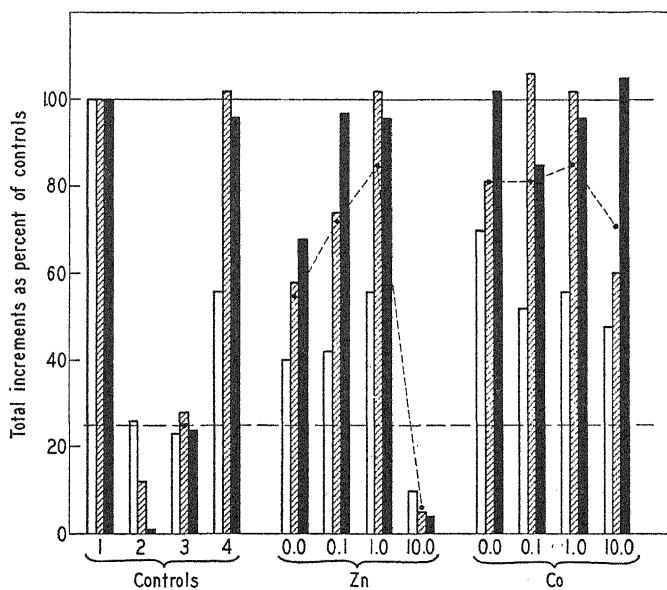


FIG. 3. Legend as for fig. 2, except that control 4 contains only I, Mn, B, Zn, and Co, without Si.

1.5 p.p.m. of  $\text{ZnSO}_4$ , while cobalt, at the concentrations tested, was without significant effect.

It thus appears that nutrients containing only four accessory salts,  $\text{MnSO}_4$ ,  $\text{ZnSO}_4$ ,  $\text{H}_3\text{BO}_3$ , and  $\text{KI}$ , are capable of maintaining growth of isolated tomato roots through at least 3 passages without apparent deterioration or diminu-

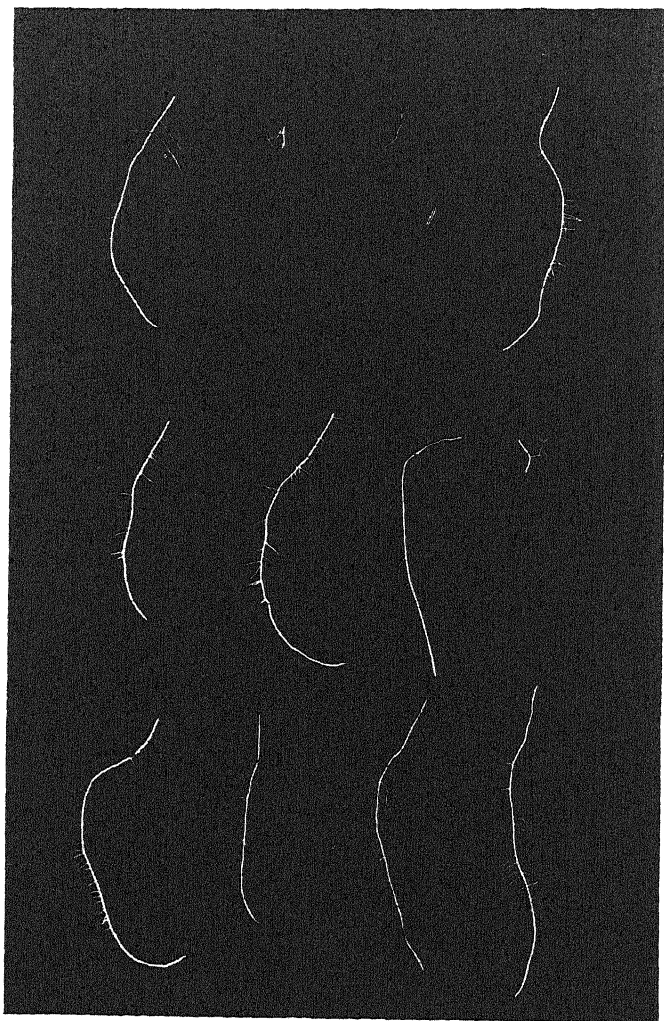


FIG. 4. Roots grown in the following solutions (reading from left to right): Top row, control with yeast extract; negative control without accessory material; nutrient containing standard salts, sugar, amino acids, and vitamin  $\text{B}_1$  but with no accessory salts; same as the last but with  $\text{Mn}$ ,  $\text{B}$ ,  $\text{I}$ ,  $\text{Zn}$ , and  $\text{Co}$  added. Middle row, nutrient the same as that in the upper right hand corner except that concentrations of  $\text{Zn}$ , 0.0, 0.1, 1.0, and 10.0 times the standard, were used. Bottom row, like the middle row but with concentrations of  $\text{Co}$  varied, instead of  $\text{Zn}$ . All pictures taken at the end of 3 passages.  $\times 0.5$ . (Photograph by J. A. CARLILE.)

tion in growth rate and at a level almost equal to that in a yeast extract medium. Addition of any of the other 8 salts originally included in the accessory salt mixture did not improve the result. As is evident from figure 4 (upper right hand corner), the condition of such roots at the end of 3 passages was excellent and the contrast between them and roots grown in a nutrient lacking these 4 salts (fig. 4, second from the right, top row) is striking. The nutrient is little if at all inferior to the control and is superior to any synthetic nutrient heretofore developed.

### Discussion

The best synthetic nutrient presented in previous work (9) contained sucrose, 18 salts, 9 amino acids, and vitamin B<sub>1</sub>. It was capable of maintaining continuous growth at a level somewhat inferior to that in a yeast extract medium. In discussing that nutrient, it was pointed out that its deficiency might be due to improper balance of the constituents already there, in which case a quantitative study might lead to correction of the deficiency, or it might be due to the absence of some desirable but not absolutely essential constituents, in which case a quantitative study would not be adequate. The present study has shown clearly that the former is, at least in large measure, the case; that is to say, the fault has been largely one of improper balance and of a *too complicated* medium. By simplifying the nutrient through the omission of 8 salts and by modifying the concentration of manganese, the results have been greatly improved and brought up to almost, if not quite, the level of the control containing yeast. The optimal synthetic nutrient developed to date then contains the following ingredients in each liter of solution:

Ca(NO <sub>3</sub> ) <sub>2</sub> .....	70.00 mg.	dl-phenylalanine .....	1.50 mg.
KNO <sub>3</sub> .....	80.00 "	dl-lysine .....	1.50 "
KCl .....	65.00 "	dl-valine .....	0.15 "
KH <sub>2</sub> PO <sub>4</sub> .....	12.55 "	dl-serine .....	0.05 "
KI .....	0.75 "	dl-isoleucine .....	0.0015 mg.
MnSO <sub>4</sub> .....	4.40 "	d-glutamic acid .....	5.00 mg.
Fe <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> .....	2.50 "	l-histidine .....	1.50 "
ZnSO <sub>4</sub> .....	1.50 "	l-proline .....	0.50 "
H <sub>3</sub> BO <sub>3</sub> .....	1.60 "	l-leucine .....	0.015 mg.
Vitamin B <sub>1</sub> —1 mg.		Sucrose—20 gm.	

It seems possible that a re-examination of the amino acid mixture, carried out in a way similar to that just described for the accessory salt mixture, may result in still further simplification of the nutrient, with concomitant improvement.

### Summary

Of the 12 accessory salts included in the formula of TRELEASE and TRELEASE, only four, MnSO<sub>4</sub>, ZnSO<sub>4</sub>, H<sub>3</sub>BO<sub>3</sub>, and KI, appear to be essential for

growth of isolated tomato roots under the conditions of the experiments here reported. Other salts may have been introduced as impurities but played no recognizable rôle in the growth of the roots in question. When the four salts named above are added to a nutrient containing the standard salts of Pfeffer's solution, iron, sucrose, amino acids, and vitamin B<sub>1</sub>, in proper proportions, the resulting nutrient is almost, if not quite, equal to a yeast extract medium as a source of materials essential for growth of these roots.

DEPARTMENT OF ANIMAL AND PLANT PATHOLOGY  
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH  
PRINCETON, NEW JERSEY

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# INFLUENCE OF FOLIAGE SPRAYS ON DROUGHT RESISTANCE OF CONIFERS

HARDY L. SHIRLEY, AND LLOYD J. MEULI

(WITH ONE FIGURE)

## Introduction

Periodic drought is one of the most serious hazards to young conifer plantations in several regions of this country. In the Lake States region, for example, droughts have occurred in four years of the past seven. Biologists have suggested that repellent substances sprayed on leaves of conifers to prevent rodent damage might, in addition, have a beneficial effect on survival during droughts. It seemed desirable to make a careful test of a few sprays to determine if they were of any real use in increasing resistance to drought. The present paper describes experiments designed to test the protective value of various sprays that have been suggested as useful agents in lowering transpiration.

## Review of literature

EMERSON and HILDRETH (2) treated ponderosa pine with four different spray materials, all of which caused a pronounced decrease in the rate of transpiration. Corn oil, the most efficient, reduced transpiration from 20.7 to 2.4 gm. daily. These sprays appeared to have no ill effects on the trees and seemed to promote increased survival of seedlings planted in the field.

MALIN (6) showed that the rate of transpiration and the osmotic pressure increased rapidly after the natural waxy coating was removed from the needles of conifers. MITTMEYER (8) found that the cuticular transpiration is very important in many plants of the xerophytic type, while for mesophytes a much larger percentage of transpiration occurs through the stomata. FÜSSER (4) showed that coating leaves with vaseline and other materials designed to reduce transpiration tended to upset their water relations to such an extent that for some leaves the transpiration rate with one surface coated with vaseline was as rapid as from the uncoated leaf. ARTHUR and STEWART (1) reported that coating leaves of tobacco plants with vaseline did not reduce transpiration to any appreciable extent when they were exposed under conditions of high light intensity and high temperature.

The effects of sprays on the water balance of plants are reflected in changes in their rate of photosynthesis. HOFFMAN (5) reported that lime-sulphur sprays decreased the rate of photosynthesis in apple leaves as much as 38 per cent. Summer oil sprays also caused a marked decrease at first, but after a week the leaves regained their initial rate of assimilation. SCHROEDER (10) found that the carbon dioxide absorption of apple leaves

was materially reduced after applications of mineral or vegetable oil sprays. WILSON and RUNNELS (14) report increases in transpiration of *Coleus* plants sprayed with copper-containing mixtures varying from 42 per cent. for colloidal copper up to 1000 per cent. for Bordeaux mixture. In an earlier report (13) these same investigators found that Bordeaux mixture checked growth, decreased yield, and increased transpiration of tomatoes, while an oil spray apparently had no unfavorable influence on these plants. ROHRBAUGH (9) found that heavy oil sprays accumulated in the leaves and twigs of citrus fruit trees where they persisted for as long as 6 months, apparently causing only slight injury to the trees. MCKAY (7), on the other hand, found that paraffin oils injured considerably the young shoots of apple trees. The evidence from the literature on the subject of protective sprays is somewhat conflicting, and might lead one to question whether foliage sprays provide sufficient protection against excessive transpiration to offset their unfavorable effects.

### Experimental methods and results

Three conifers, Norway pine, *Pinus resinosa* Solander, white pine, *Pinus strobus* L., and white spruce, *Picea glauca* (Moench) Voss., were used in these tests.

Sprays, furnished by the Central Great Plains Horticulture Field Station at Cheyenne, Wyoming, were made up as follows:<sup>1</sup>

#### No. 1:

Beeswax .....	10 parts (weight)
Fish oil soap .....	1 part
Water .....	50 parts

Dissolve the soap in water by heating to 95° C.; melt the beeswax and heat to 70° C.; add beeswax *slowly* to heated soap solution while stirring vigorously.

#### No. 2:

Parawax .....	5 parts (weight)
Beeswax .....	5 parts
Fish oil soap .....	1 part
Water .....	40 parts

Prepare the same as no. 1.

#### No. 3:

Goodrich rubber solution .....	1 pound
Fish oil soap .....	2 ounces
Water .....	2 ounces

Dissolve the soap in water and cool to room temperature. Add the rubber solution to the soap solution while stirring vigorously.

#### No. 4:

Hydrowax liquid N full strength.<sup>2</sup>

<sup>1</sup> The writers are indebted to J. L. EMERSON, formerly of the Central Great Plains



## LABORATORY TESTS

Laboratory tests of drought resistance were made in the 1933 drought machine (11). Two-year-old white spruce seedlings were potted in tin cans and allowed a week or more to become established. The materials were sprayed on the leaves with a hand sprayer, just before placing the plants in the testing machine. The soil in all plant containers was maintained at the same moisture content by daily additions of water through a glass pipette in the waxy seal. The plants remained in the machine until dead. An untreated control was tested with each lot of sprayed plants.

A measure of the reduction in transpiration might be considered a justifiable estimate of spray values during periods of drought. Consequently records of daily water loss from each plant were kept for the three laboratory tests as shown in figure 1. It will be noted that the more effective sprays

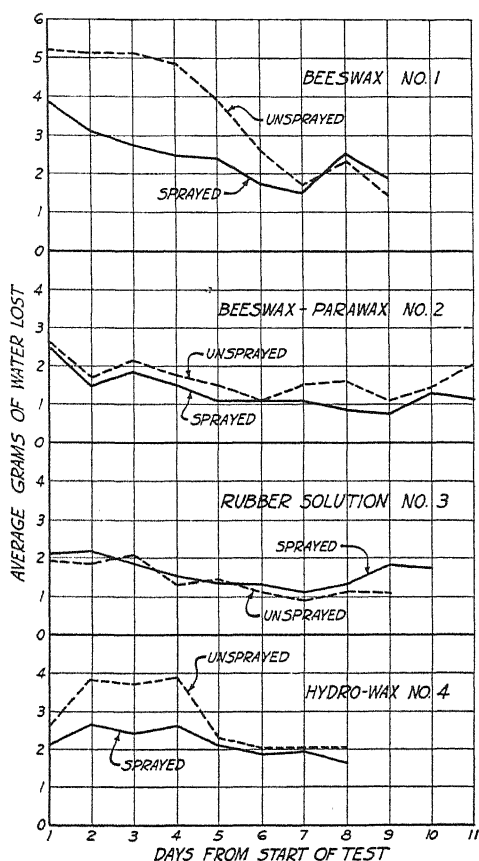


FIG. 1. Effect of foliage sprays in daily transpiration of white spruce.

Horticulture Field Station at Cheyenne, Wyoming, for the sprays used in these tests.

<sup>2</sup> Purchased from Glyco Products Co., Brooklyn, New York.

TABLE I

EFFECT OF SPRAYS ON DROUGHT RESISTANCE OF WHITE SPRUCE. AVERAGE OF  
3 LABORATORY TESTS

SPRAY USED	TOTAL NUMBER OF PLANTS SPRAYED AND UNSPRAYED	AVERAGE DAYS SURVIVED IN MACHINE	
		SPRAYED	CONTROL
(1) Beeswax .....	86	8.34	8.56
(2) Beeswax-parawax .....	86	9.10	8.93
(3) Rubber solution .....	81	8.60	8.84
(4) Hydrowax .....	85	8.63	9.50

caused an important reduction in transpiration during the first four days, but thereafter, as the available soil moisture was reduced, unsprayed and sprayed plants lost water at approximately the same rate.

TABLE II

ANALYSIS OF VARIANCE OF DAYS SURVIVAL IN DROUGHT MACHINE BY SPRAYED AND  
UNSPRAYED SPRUCE SEEDLINGS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	OBSERVED F
Spray 1			
Total	85	9.15	
Between treatments .....	1	6.15	1.01
Between tests .....	2	137.29	22.64*
Remainder .....	82	6.06	
Spray 2			
Total	85	7.98	
Between treatments .....	1	.94	.27
Between tests .....	2	199.13	58.26*
Remainder .....	82	3.42	
Spray 3			
Total	80	11.00	
Between treatments .....	1	5.31	1.62
Between tests .....	2	310.97	94.84*
Remainder .....	77	3.28	
Spray 4			
Total	84	18.73	
Between treatments .....	1	32.80	6.17†
Between tests .....	2	555.11	104.43*
Remainder .....	81	5.32	

\* Indicates significance beyond 0.01 level.

† Indicates significance between 0.05 and 0.01 levels.

**TABLE III**  
EFFECT OF FOLIAGE SPRAYS ON FIRST-YEAR MORTALITY OF PLANTED CONIFERS

SPRAY TREATMENT	AVERAGE MORTALITY					
	PIKE BAY EXPERIMENTAL FOREST*			CUTFOOT EXPERIMENTAL FOREST*		WEIGHTED MEAN ALL SPECIES
	LOT 1 NORWAY PINE 22 GROUPS	LOT 2 WHITE PINE 5 GROUPS	LOT 3 WHITE SPRUCE 18 GROUPS	LOT 4 NORWAY PINE 12 GROUPS	LOT 5 WHITE SPRUCE 8 GROUPS	
Untreated .....	% 8.21	% 0.00	% 8.05	% 11.22	% 14.30	% 8.84
(1) Beeswax .....	10.50	3.09	7.12	12.64	14.70	9.90
(2) Beeswax-parawax .....	8.24	1.75	11.80	12.35	19.30	11.03
(3) Rubber solution .....	9.02	2.19	12.80	12.89	26.08	12.39
(4) Hydrowax .....	9.22	4.63	12.70	14.28	22.11	12.37
						% ..... 1.06 2.19 3.55 3.53

\* On the Chippewa National Forest, Minnesota.

† The standard error of these differences is 1.375. Values of 2.75 or greater are considered to be significant and are designated by bold-faced figures.

Data from all tests were grouped together (table I) and treated by FISCHER's (3) analysis of variance method.<sup>3</sup>

The results are given in table II. It will be noted that differences between tests are highly significant. This was due very likely to the different stages of vegetative development at the time of testing. In only one case is there a significant difference between treated and control plants. Hydrowax liquid N caused a reduction in drought resistance and at the same time caused a considerable decrease in transpiration. Presumably its lowering of drought resistance may be attributed to effects other than those effecting the transpiration rate. All sprays caused the needle tips to turn brown.

During 1937 a new paraffin emulsion appeared, designed to protect plants from desiccation.<sup>4</sup> A sample of this material obtained from the Dow Chemical Company, Midland, Michigan, was diluted with water 1 to 3½ and sprayed on 2-year-old jack pine. A machine test of drought resistance, involving 45 treated and 45 control plants, was made in the usual manner. Both treated and control plants lived an average of 4.9 days.

#### FIELD TESTS OF DROUGHT RESISTANCE

Field tests were made in two different areas to determine the influence of sprays on field survival. These field test areas, about thirty miles apart, were located in the Chippewa National Forest. During the test period in 1934, precipitation at Cass Lake, Minnesota, was about normal in June, 1.34 inches below normal in July, and 1.87 inches below normal in August. The drought was not quite as severe as those which occurred in other years of the past seven.

A total of 49,000 plants consisting of the three species, Norway pine, white pine, and white spruce, were used in the tests. The plants were sprayed in bundles before planting in the field. At the end of the first growing season 50 per cent. of the planted trees were examined to determine the percentage of mortality. The results of these counts are given in table III. In general, spraying resulted in increased mortality. The only exception was white spruce which, when sprayed with beeswax, had a lower mortality

TABLE IV  
ANALYSIS OF VARIANCE OF FIELD MORTALITY OF SPRAYED AND UNSPRAYED CONIFERS

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	OBSERVED F
	Total 324		
Within lots (error) .....	300	61.57	
Between lots .....	4	1323.46	21.50*
Between treatments .....	4	154.14	2.50†
Interaction, lots × treatments .....	16	55.79	0.91

\* Indicates significance at 0.01 level (12).

† Indicates significance at 0.05 level (12).

<sup>3</sup> Assistance with statistical treatment was furnished by R. H. BLYTHE, JR.

<sup>4</sup> MILLER, E. J., NEILSON, J. A., and BANDEMER, S. L. Wax emulsions for spraying nursery stock and other plant materials. Michigan Agr. Exp. Sta. Tech. Bull. 282. 1937.

than unsprayed plants. This advantage occurred only on the Pike Bay Experimental Forest. These data were treated by FISHER's (3) analysis of variance method as shown in table IV. Differences between lots and between treatments were both significant. The standard error for the mean of all species and sites was computed to determine which particular treatments differed significantly from the untreated. The increase in average mortality over that of untreated plants is also shown in table III.

In field tests, plants treated with both rubber solution and Hydrowax had significantly higher mortality than untreated plants. These sprays must be considered distinctly harmful under the conditions used. The other two sprays were not beneficial.

### Discussion of results

Neither in the laboratory tests nor in field survival did sprayed plants exhibit any significant advantage over untreated ones. In the laboratory tests the plants were subjected to severe atmospheric and soil drought. In field tests the drought was only moderate. In view of the rather extensive trial given, it appears logical to assume that none of the four sprays afforded any real protection against drought to the species used. In this region, at least, their effect, if any, was detrimental. Whatever benefit that may have resulted in reducing transpiration of the sprayed plants was evidently offset by disadvantages due to other causes.

### Summary

1. Norway pine, white pine, and white spruce were treated with four foliage sprays to determine their efficacy in improving resistance to drought as measured by laboratory and field tests.

2. None of the sprays studied improved resistance to drought in these conifers.

LAKE STATES FOREST EXPERIMENT STATION  
ST. PAUL, MINNESOTA

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# FORMATION OF NITRATE IN DETACHED GREEN LEAVES OF SWISS CHARD AND TOMATO

MARY C. MCKEE AND DOROTHY E. LOBB

(WITH TWO FIGURES)

## Introduction

In 1933, VICKERY, PUCHER, WAKEMAN, and LEAVENWORTH (6) reported the formation of nitrate in tobacco leaves subjected, in the absence of light, to either air drying or distilled water culture. The present investigation seeks to extend this unprecedented (1) observation to leaves other than those of the tobacco; for this purpose random choice was made of Swiss chard (*Beta vulgaris* var. *cicla*) and tomato *Lycopersicum esculentum* Mill). The treatment of the leaves followed as closely as possible that given the tobacco; the results so obtained indicate that the tobacco is not unique in showing nitrate formation and that the phenomenon differs in magnitude depending upon the nature of the leaf and its condition.

## Procedure

### SWISS CHARD

PREPARATION OF DRIED LEAF TISSUE.—Two series of investigations were carried out on Swiss chard (var. *Lucullus*) at the end of three months' growth. The two crops were grown from the same seed, at different seasons of the year, in different locations, and under different nutritive conditions.

Lot A was planted in July, 1936, in an open field and received little cultivation. The leaves were harvested in October. Lot B was planted January 16, 1937, in the college greenhouse. Once a week from the second week after planting until ten days before picking, April 5, the twenty-four plants received a pint of nutrient solution of the following composition: calcium nitrate, 4 gm.; potassium nitrate, 1 gm.; magnesium sulphate, 1 gm.; potassium dihydrogen phosphate, 1 gm.; potassium chloride, 0.5 gm.; water, 1 liter.

In each case the leaves were harvested about eight o'clock in the morning. They were practically free from surface moisture and were brushed free from soil. All except very old and very young, green, healthy leaves were stripped from the plants and, therefore, the samples were of varied sizes and stages of development. The petioles were removed unless the leaves were to be water cultured, the leaves were divided into groups of approximately 100 gm. each, washed in tap water, blotted dry, and treated as follows:

*Controls.*—Groups A-1 and B-1 were shredded, piled lightly on pyrex plates and dried in an electric oven for five to six hours at 75° to 100° C. Frequent turning of the leaves aided in promoting uniform drying. At the end of this period the leaves were allowed to cool in the air, weighed, ground in a Nixtamal mill or by hand in a glass mortar, and bottled for later determination of nitrate and total solids.

*Water culture in the dark.*—Leaves of groups A-2, A-3, A-4, B-2, B-3, B-4 were placed in liter beakers with the petioles dipping in distilled water and kept in a dark room at approximately 24° C. for periods of about 48, 72, and 96 hours. Fresh water was supplied and the stems scraped each twenty-four hours. The leaves were turgid and there was no visible evidence of bacterial action. At the end of each culture period the petioles were removed and the leaves oven-dried, weighed, and ground. The weight was corrected for the removal of the petioles.

*Air drying in the dark.*—Leaf groups A-5, A-6, A-7, B-5, B-6, B-7 were suspended by cords passing through the mid rib of each leaf, in a well ventilated dark room where they dried slowly at 20° to 22° C. for three-, six-, and nine-day periods respectively. Following the curing process, each lot was dried in the oven for three to four hours. This left the leaves a bright green color in contrast to the controls and water cultured leaves which were darkened by oven drying. The dried leaves were air cooled, weighed, ground, and preserved in glass stoppered bottles.

**ANALYTICAL PROCEDURE.**—Determination of total solids in (a) fresh leaf and (b) dried leaf tissue. (a) Two fresh leaves were shredded and weighed quantitatively in no. 4 porcelain crucibles, oven heated with the controls, transferred from the oven to a vacuum desiccator and dried to constant weight over sulphuric acid (sp. gr. 1.84). The percentage of total solids in fresh leaf as obtained in this way was checked by following the change in weight upon drying of the different batches of leaves.

(b) Duplicate samples (one to two gm. each) of the bottled oven dried leaf material were dried to constant weight *in vacuo* over sulphuric acid (sp. gr. 1.84).

*Determination of nitrate-nitrogen in the oven dried leaf.*—Following the technique of PUCHER, VICKERY, and WAKEMAN (3), one- to two-gram samples of the oven dried leaf powder were brought to a pH just less than 1 (quinhydrone method) by addition of 4 N sulphuric acid, and the nitric acid so formed was removed by extraction with alcohol- and peroxide-free ether in an underwriter's extraction apparatus. Complete removal of the nitrate ion was tested by use of diphenylamine. The ether extract was diluted with water, neutralized with 0.5 N sodium hydroxide, the ether removed by evaporation, and the aqueous solution made up to 100 ml. Aliquots of 5 or 10 milliliters were reduced with iron powder and sulphuric acid and the ammonia determined by distillation from an alkaline solution into hydrochloric acid and subsequent Nesslerization. A Duboseq colorimeter was used and corrections were made for the nitrogen content of the iron powder and the ammonia content of the leaf ether extract.

#### TOMATO

Tomato seed (var. Margrove) was planted, April 4, 1936, in the college greenhouse; the young plants were removed to an outdoor garden on May



18. The plants were well fertilized with nutrient solution (described under Swiss chard) and sheep manure, and they grew vigorously. Two lots of clean, healthy, green leaves of varied sizes and ages were harvested, one on the morning of July twenty-second, the other on the afternoon of August eighth. They were treated in a similar manner and subjected to the same analyses as the chard leaves.

### Results

The experimental data are summarized in tables I and II. A comparative study of the results obtained with tobacco (VICKERY *et al.*), Swiss chard, and tomato leaves may be made by means of figures 1 and 2.

### Discussion

In the analysis of green leaves two difficulties are always met, the procuring of a uniform sample of the fresh leaf and the preparation of representative material for direct analysis. The leaves used in this work were of varied sizes and stages of development and the attempt to surmount the first difficulty lay solely in the use of a large number of leaves well mixed. In table II, under A-1, are recorded the analyses of three separate groups of unselected leaves all subjected to the same experimental procedure. The

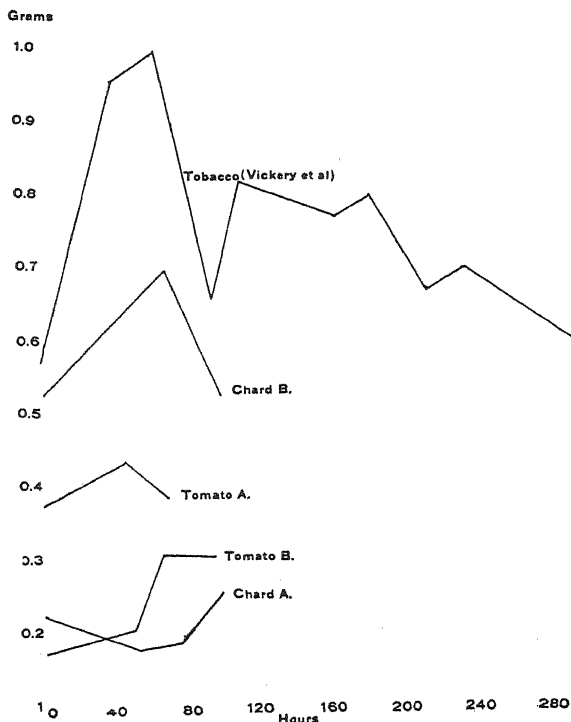


FIG. 1

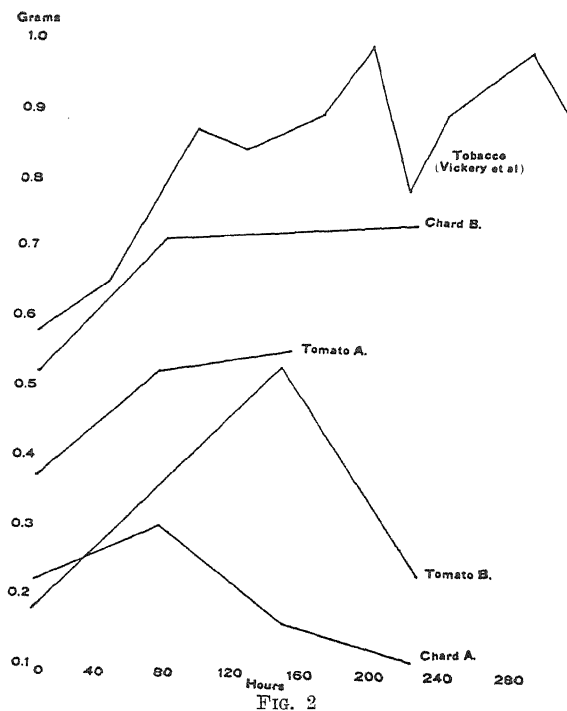


FIG. 2

TABLE I

NITRATE CONTENT OF SWISS CHARD LEAVES

SAMPLE	NITRATE-N IN ONE GRAM OF OVEN DRIED LEAF	TOTAL SOL- IDS IN 100 GRAMS OF OVEN DRIED LEAF	NITRATE-N IN ONE GRAM OF TOTAL SOLIDS	TOTAL SOL- IDS IN 100 GRAMS OF FRESH LEAF	NITRATE-N IN ONE GRAM OF FRESH LEAF
	mg.	mg.	mg.	mg.	mg.
A					
Control, A-1 .....	1.92	94.8	2.03	11.3	0.229
Water cultured					
48 hr., A-2 .....	1.54	94.2	1.63		0.184
70 hr., A-3 .....	1.58	94.6	1.63		0.189
92 hr., A-4 .....	2.11	95.2	2.22		0.250
Air dried					
72 hr., A-5 .....	2.54	94.2	2.69		0.305
144 hr., A-6 .....	1.48	93.6	1.56		0.176
216 hr., A-7 .....	1.04	94.2	1.11		0.125
B					
Control, B-1 .....	7.24	95.5	7.50	7.06	0.530
Water cultured					
48 hr., B-2 .....	8.92	97.7	9.14		0.645
72 hr., B-3 .....	9.70	97.5	9.95		0.703
96 hr., B-4 .....	7.54	98.3	7.67		0.541
Air dried					
72 hr., B-5 .....	9.80	97.1	10.1		0.712
144 hr., B-6 .....	9.71	95.4	10.2		0.719
216 hr., B-7 .....	9.87	94.5	10.5		0.737

TABLE II  
NITRATE CONTENT OF TOMATO LEAVES

SAMPLE	NITRATE-N IN ONE GRAM OF OVEN DRIED LEAF	TOTAL SOL- IDS IN 100 GRAMS OF OVEN DRIED LEAF	NITRATE-N IN ONE GRAM OF TOTAL SOLIDS	TOTAL SOL- IDS IN 100 GRAMS OF FRESH LEAF	NITRATE-N IN ONE GRAM OF FRESH LEAF
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
A					
Control, A-1 .....					
a. ....	3.35	91.5	3.66	10.52	0.385
b. ....	3.31	94.1	3.52	10.54	0.371
c. ....	3.05	88.9	3.43	11.28	0.387
				10.8 av.	
Water cultured					
43 hr., A-2 .....	3.88	94.8	4.09	10.8	0.442
64 hr., A-3 .....	3.54	95.6	3.70		0.400
Air dried					
72 hr., A-5 .....	4.56	93.7	4.87		0.526
144 hr., A-6 .....	4.99	95.5	5.23		0.564
B					
Control, B-1 .....	1.51	91.7	1.64	11.8	0.195
Water cultured					
45 hr., B-2 .....	1.54	89.5	1.72		0.203
66 hr., B-3 .....	2.37	90.0	2.64		0.311
90 hr., B-4 .....	2.37	89.7	2.64		0.311
Air dried					
66 hr., B-5 .....	2.51	91.0	2.76		0.326
138 hr., B-6 .....	4.15	91.1	4.56		0.538
220 hr., B-7 .....	1.97	93.8	2.10		0.248

results show that the differences in values for nitrate and total solid content are well within the range of experimental error and the conclusion that a mixture of many leaves yields fairly uniform samples seems justified.

Many observations (2, 4, 5, 7, 8, etc.) confirm the selective storage of nitrates in different structural parts of a plant. It is not possible to insure a similar weight ratio of blade, rib, and petiole in all groups of leaves. Thorough mixing of the ground dried material from a large number of leaves was the method used in meeting this second source of error.

While it is probable that no nitrate was lost in drying at the temperature used, the same assumption cannot be made in regard to volatile oils and, perhaps, to chemically combined water. It may well be that the weight of the total solids varies depending upon whether the leaves are, or are not, air dried before heating. To test this, two one-leaf samples of Swiss chard (lot B) were weighed in duplicate; one was oven heated immediately for five hours, then dried to constant weight in a vacuum desiccator over concentrated sulphuric acid; the other was air dried for three days before it was heated and dried *in vacuo*. The values for the weight of total solids in 100 grams of fresh leaf, as obtained in these two ways, were 7.06 and 7.36 grams. No increased loss of weight had resulted from the exposure of the chard leaves

to the air for three days before heating. A similar check was not made with tomato leaves.

### Summary

Swiss chard and tomato leaves when subjected to air drying at room temperature in a well ventilated place showed, for a limited time, an increase in nitrate content. The change was more pronounced in the tomato than in the chard and varied in two lots of leaves grown from the same seed but under different conditions.

Both Swiss chard and tomato leaves when cultured in distilled water, in absence of light, gave evidence of slight nitrate formation. Again the increase in nitrate varied with the nature and condition of the leaf.

These observations are in agreement with that of VICKERY *et al.* who, in 1933, reported an increase in the nitrate content of tobacco leaves during the early stages of curing and water culture.

The writers wish to acknowledge their indebtedness to Dr. H. B. VICKERY and Dr. G. W. PUCHER for advice in carrying out this investigation.

DEPARTMENT OF CHEMISTRY  
CONNECTICUT COLLEGE  
NEW LONDON, CONNECTICUT

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# FORMATION OF CAROTENOIDS AND CHLOROPHYLLS IN ETIOLATED BARLEY SEEDLINGS EXPOSED TO RED LIGHT

HAROLD H. STRAIN

(WITH ONE FIGURE)

## Introduction

For the investigation of many of the problems pertaining to the metabolism and function of the leaf pigments, it would be desirable to vary the relative proportions of the several leaf pigments in any given plant through changes in the environment. Average pigment determinations reported by RUDOLPH (1) have already indicated that red light, which promotes the formation of chlorophyll, does not stimulate the formation of the yellow pigments in etiolated bean leaves, but the individual determinations were subject to great variation. Moreover, etiolated bean leaves contain relatively large quantities of carotene and xanthophylls in nearly the same proportions found in green leaves, so that, for demonstration of the effect of red light on the formation of carotenoids in these seedlings, extremely precise determinations of the pigments were required.

Investigations in this laboratory have shown that etiolated barley leaves, in contrast with the etiolated bean seedlings, contain very small quantities of carotene relative to the xanthophylls. These investigations also made possible the precise determination of the carotenoid pigments, because it was found that the rapid oxidation of the yellow pigments which takes place when the etiolated leaves are ground with sand and acetone during the extraction of the pigments may be prevented by treatment of the leaves with hot water (4). By the use of the improved methods of analysis, it was possible to determine accurately the effect of red light on the formation of the carotenoids in etiolated barley plants.

When etiolated barley seedlings are exposed to red light, both carotene and chlorophylls increase rapidly. The xanthophylls, comparatively large quantities of which are present in the etiolated barley seedlings, increase more slowly. Investigation of the pigments of etiolated barley seedlings, which had been impregnated with sucrose and kept in the dark, demonstrated that the presence of sucrose did not promote the formation of carotene or chlorophyll. Determination of the absorption spectra of lutein acetate and of beta-carotene demonstrated that lutein and beta-carotene, the principal constituents of the leaf carotenoids, absorb considerable light in the red region of the spectrum in which chlorophyll exhibits maximum absorption. Since etiolated barley seedlings contain extremely little green

pigment, the red light absorbed by the yellow pigments in these plants must represent a large proportion of that absorbed by all the pigments that are present. Whether the formation of the carotene in red light is dependent upon the absorption of light by the yellow pigments or upon other factors remains to be established.

## Experimentation

### PLANT MATERIAL

Etiolated barley seedlings were obtained by germinating barley seeds in 6-inch square boxes in the dark room. All the seedlings were grown in the same soil in order to insure comparable nutritional conditions.

### APPARATUS

The apparatus for the illumination of the seedlings was constructed from a metal can  $13 \times 9 \times 9$  inches. Light of the desired wave length was admitted to the can through two  $6\frac{1}{2}$ -inch square glass filters set in modeling clay over openings in the top of the can. A 100-watt, inside-frosted tungsten lamp was placed 3 inches above each of the glass filters. Ventilation for the light chamber was provided by means of flexible metal tubing  $1\frac{1}{2}$  inches in diameter and  $3\frac{1}{2}$  feet long soldered to openings in each end of the can. One tube was bent upward in the shape of an *S*; the other was bent downward

TABLE I  
TRANSMISSION OF LIGHT BY RED GLASS FILTERS

FILTER I		FILTER II	
WAVE LENGTH	TRANSMISSION	WAVE LENGTH	TRANSMISSION
<i>mμ</i>	%	<i>mμ</i>	%
590	0.00	627.5	0.00
595	0.17	630	0.14
600	1.8	632.5	0.27
605	9.7	635	0.87
610	24.4	637.5	2.28
615	39.7	640	6.1
620	48.9	642.5	12.5
625	55.4	645	21.7
630	58.8	647.5	32.4
640	61.6	650	43.8
650	63.8	655	58.3
660	65.8	660	64.8
670	65.3	670	71.5
680	67.2	680	72.4
		690	73.3
		700	74.4

in the shape of an *S*. In order to prevent direct admission of light to the tubing, black baffles, 6 inches square, were placed 3 inches from the open ends. Temperature within the light chamber varied from 21 to 26.6°.

Typical spectral transmissions of the two types of glass filters used to obtain red light are shown in table I. These values were subject to some variation due to imperfections in the surface and to variation in the thickness of the glass. The transmission curves and the absorption of light by solutions of the pigments extracted from the leaves were determined with the photoelectric spectrophotometer described by SMITH (2).

In order to make certain that the etiolated plants were exposed only to light of the wave lengths transmitted by the red filter, blue glass filters were placed over the red glass filters, and etiolated plants were grown in the light chamber while the tungsten lamps were allowed to burn continuously. After several days, the plants did not contain increased quantities of carotene or green pigments. This indicated that stray light was not entering the light chamber and that blue light was not transmitted by the red glass filters.

#### DETERMINATION OF PIGMENTS

Samples of the leaves (5 gm.) were placed in water at 95–100° for 1 minute. The water was separated by decantation and the leaves were ground with sand (12.5 gm.) and a little acetone. The ground mass was placed on a filter consisting of heat-treated siliceous earth supported on cotton, and the solvent was removed from the leaf material with suction. The residue was then washed with acetone until all the pigments had been removed. After the addition of ether (75 ml.) to the filtrate containing the pigments (75 to 100 ml.), the pigments were transferred to the ether by further dilution of the solution with water (about 200 ml.). The aqueous layer was separated from the ether and re-extracted with fresh ether (40 ml.). The combined ether layers were washed well with water and rinsed into a volumetric flask (100-ml.) with ethanol. An aliquot portion of this solution was diluted several times with ethanol, and the absorption of the solution was determined. Another aliquot portion of the solution of pigments was treated with a solution of potassium hydroxide in methanol in order to saponify the chlorophylls. The saponified chlorophylls were separated from the yellow pigments by washing the solution with water after the addition of some ether. The ether solution of the carotenoids was transferred to a volumetric flask and diluted to volume with ethanol. An aliquot portion of this solution was diluted with ethanol and the absorption was determined. Another aliquot portion of the solution of the carotenoids was diluted with petroleum ether, and the pigments were transferred to the petroleum ether by the addition of water to the solution. The petroleum ether solution was then extracted successively with 60, 70, 80, and 90 per

cent. methanol in order to remove the ether and the xanthophylls. The petroleum ether solution of the carotenes was diluted to a definite volume with ethanol, and the absorption was determined.

Experience with the leaf xanthophylls had demonstrated that leaves contain a mixture of these pigments which differ from one another with respect to their spectral absorption properties (3). In order to compare the xanthophyll, carotene, and chlorophyll content of leaves which had been permitted to develop under various conditions, it was necessary to calculate the spectral absorption coefficients of the pigments per gram of leaf material per liter of extract. In order to prevent confusion with  $E$ , the symbol for the absorption coefficient per gram of pigment per liter of solution, the following symbols have been used:

$$s = (\log I_0 - \log I) (1/Lc)$$

where  $I_0$  and  $I$  are respectively the transmission of  $L$  cm. (2 cm.) of solvent and solution. The concentration,  $c$ , is expressed in grams of leaf material per liter of extract. Since  $s(\text{total pigments})$ ,  $s(\text{total carotenoids})$ , and  $s(\text{carotene})$  were determined experimentally,

$$s(\text{chlorophyll}) = s(\text{total pigments}) - s(\text{total carotenoids}),$$

and

$$s(\text{xanthophyll}) = s(\text{total carotenoids}) - s(\text{carotene}).$$

### Results

The results of several experiments are summarized in table II. In experiment I, the pigments of etiolated barley seedlings 12 days old were determined. In experiment II, etiolated barley seedlings 5 days old were exposed continuously for 89 hours to light from filter I. For experiment III, the seedlings were exposed continuously for 168 hours to light from filter I. The plants for experiment IV were exposed continuously for 48 hours to light which passed through two glasses of filter II. In experiment V, the etiolated seedlings were exposed continuously for 96 hours to light which passed through two glasses of filter II.

The pigment content of etiolated barley seedlings which had been impregnated with 7.5 per cent. sucrose solution is recorded under experiment VI in table II. The etiolated seedlings, 9 days old, were cut so that the leaves were about 3 inches long. These leaves were impregnated with the sucrose solution by alternately exposing the leaves to vacuum in the presence of the sugar solution and then releasing the vacuum. The impregnated leaves were permitted to stand in the dark with their cut ends in a little of the sucrose solution. After 15 hours the leaves were removed from the solution, killed in hot water, and the relative proportions of the carotenes and xanthophylls were determined.



TABLE II  
FORMATION OF PIGMENTS IN ETIOLATED BARLEY SEEDLINGS EXPOSED  
TO VARIOUS CONDITIONS

LENGTHS WAVE	PIGMENTS	ABSORPTION COEFFICIENTS IN EXPERIMENTS					
		I	II	III	IV	V	VI
$m\mu$		<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>	<i>s</i>
440	Carotene	0.0010	0.0085	0.0078	0.0068	0.0071	0.0016
	Xanthophyll	0.0177	0.0260	0.0239	0.0199	0.0196	0.0217
	Chlorophyll	0.0003	0.0415	0.0360	0.0248	0.0311	.....
455	Carotene	0.0010	0.0096	0.0087	0.0078	0.0081	0.0016
	Xanthophyll	0.0139	0.0209	0.0188	0.0157	0.0158	0.0177
	Chlorophyll	0.0001	0.0195	0.0182	0.0101	0.0155	.....
465	Carotene	0.0009	0.0083	0.0076	0.0066	0.0068	0.0013
	Xanthophyll	0.0138	0.0214	0.0189	0.0166	0.0159	0.0174
	Chlorophyll	0.0000	0.0208	0.0192	0.0110	0.0161	.....
475	Carotene	.....	0.0087	0.0077	0.0069	0.0071	0.0014
	Xanthophyll	.....	0.0217	0.0196	0.0171	0.0163	0.0196
	Chlorophyll	.....	0.0154	0.0130	0.0070	0.0118	.....
662	Chlorophyll	.....	0.0467	0.0437	0.0301	0.0373	.....
664	Chlorophyll	0.0000	0.0473	0.0447	0.0302	0.0375	0.0000
666	Chlorophyll	.....	0.0453	0.0422	0.0295	0.0356	.....

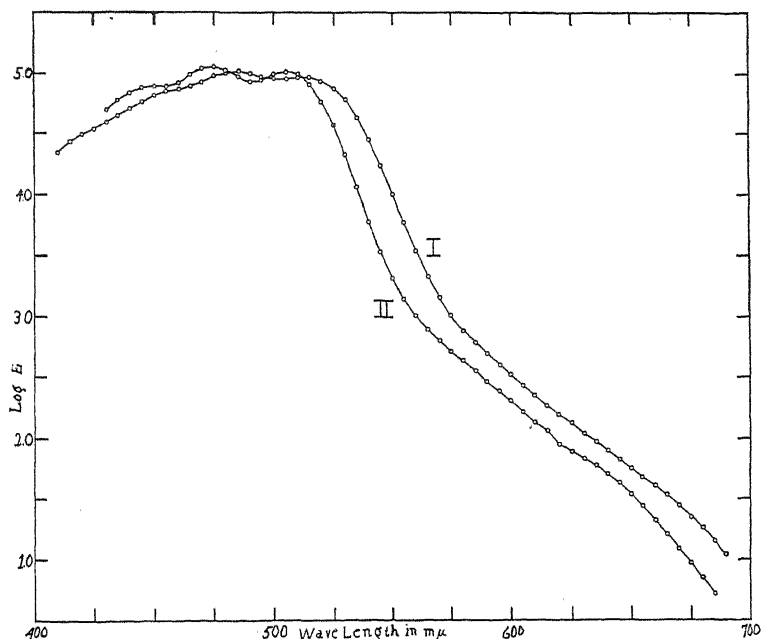


FIG. 1. Molecular absorption spectra of  $\beta$ -carotene, curve I; of lutein acetate, curve II. Solvent is carbon disulphide.

Molecular, spectral absorption curves of beta-carotene and of lutein acetate in solution in carbon disulphide are shown in figure 1. Since it was impossible to obtain a sufficiently concentrated solution of lutein in carbon disulphide for the determination of the absorption coefficients in the red region of the spectrum, it was necessary to use the more soluble acetate. This ester of lutein exhibits molecular absorption coefficients identical with those of lutein in the spectral region from 400 to 530 m $\mu$ . The molecular absorption coefficients were calculated according to the formula:

$$\log E = \log [(\log I_0 - \log I) (1/Lc)]$$

where the concentration, *c*, is expressed in moles per liter of solution. Even considering the shift in the absorption spectra of the carotenoids caused by carbon disulphide (2), these pigments absorb considerable light in the red region of the spectrum in which chlorophyll exhibits maximum absorption.

CARNEGIE INSTITUTION OF WASHINGTON  
STANFORD UNIVERSITY, CALIFORNIA

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# A PHOTOELECTRIC PHOTOMETER FOR CHEMICAL ANALYSIS

B. N. SINGH AND N. K. ANANTHA RAO

(WITH THREE FIGURES)

## Introduction

Colorimetric methods have in recent years assumed considerable importance in chemical analytic procedures. The advantages inherent in these methods are their extraordinary simplicity, expeditious nature, and the possibility of measuring quantitatively with their aid concentrations which are measurable only with great difficulty, if at all, by gravimetric methods. On the other hand certain inherent imperfections of the method need elimination. Some of the sources of error are the disparities in color of standard solutions, the necessity for dilution at higher concentrations, and the dependence on the ability of the eye to match varying depths of color, an operation in which the human eye is notoriously inefficient. Moreover color blindness, a defect not uncommonly met, stands as a serious draw-back in the use of these methods.

The frequent necessity of making a large number of quantitative estimations of sugars and other substances in physiological researches has repeatedly called for simplifying the procedure so as to eliminate the possibilities of personal error in observations, to detect more minute differences than is possible by preceding methods, and to be applicable to varied purposes. With this object in view the apparatus described here was developed in this laboratory and has been in use for some time.

## Principle

The general principle of the apparatus is similar to the one that has been developed in this laboratory for chlorophyll estimation (3). In devising this apparatus for the chemical analysis of sugars and other organic and inorganic substances, use has been made of the principle of determining the concentration of the substance occasioning a definite color reaction by the measurement of light absorption in the solution within an appropriately selected region of the spectrum. Following the well-known LAMBERT-BEER law—"The negative logarithms of the transmitted light intensities  $K$  (extinction coefficient) at equal stratum thickness bear the same proportion to each other as the concentrations,  $C$ , of the dissolved substances,"—the concentration in any particular case is calculated from the equation

$C_2 = C_1 \frac{K_2}{K_1}$ . When once a series of measurements is recorded *in the same region of the spectrum*, on a suitably graduated range of solutions having known concentrations, a calibration graph is obtained from which the con-

centration corresponding to any observational reading may then be read off directly.

### Apparatus

The apparatus is shown in figure 1. A constant source of light,  $L$ , supplies two equal beams of light which fall on the absorption vessels,  $V_1$  and  $V_2$ . The transmitted lights from the absorption vessels pass through the

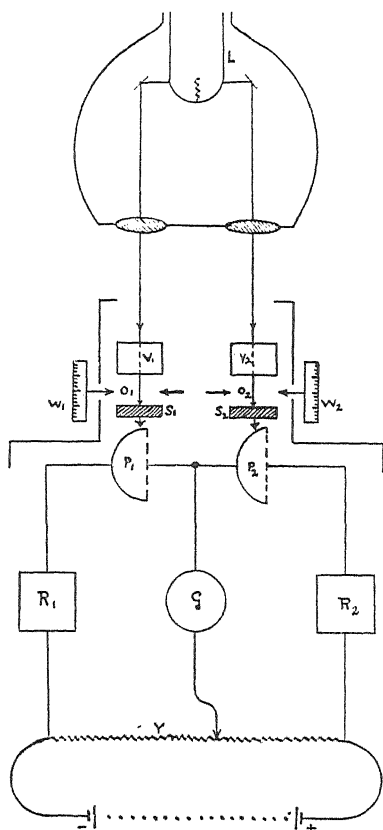


FIG. 1. Diagram of photoelectric photometer.

openings,  $O_1$  and  $O_2$ , whose sizes can be measurably varied.  $S_1$  and  $S_2$  are two slots for the reception of the spectral filters within the range of which a particular measurement is to be made. The transmitted lights from the two sides then fall on the two photoelectric cells,  $P_1$  and  $P_2$ , respectively (Osram photocell, Caesium. C. G. I. Becker and Co.), which emit currents in direct proportion to the light energies absorbed on their faces. The photoelectric emission produced by two beams of the same quality and quantity is the same, and hence if the quality is unchanged and the quantity alone varies, the rate

of current emission would be proportional to the quantity of light. A compensating device which converts direct reading into null-method is employed, the balance being effected by adjusting the lights that alter the photoelectric currents. The circuit is essentially of the bridge type,  $R_1$  and  $R_2$  being the protective resistances (10,000 ohms), and  $G$  a sensitive null-point galvanometer. The differences, if any, in the two cells are eliminated by the use of the common battery shunted by  $r$ .

The *caesium cell* is specially selected on account of its even sensitivity in response to light over the whole visible range.

Figure 2 illustrates the light diminution arrangement. Each of the

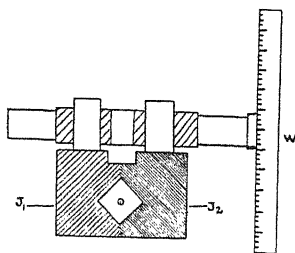


FIG. 2. Arrangement for diminution of light.

openings,  $O_1$  and  $O_2$ , are formed by two rectangular V-shaped jaws,  $J_1$  and  $J_2$ . The latter can be moved, one above the other, either towards or away from each other, in such a way that the centre of the aperture always coincides with the optic axis. The ratio of the two amounts of light transmitted is proportional to the ratio of the two areas of the apertures. The graduations<sup>1</sup> etched on the recorder,  $W$ , give a measure of the transmitted light as a percentage of the incident light. The scale on the recorder represents 100 as the largest opening and 0 the smallest.

The measurement is made within the range of a narrowly defined region of the spectrum which is furnished by selective spectral filters, dividing the visible spectrum into eight equal parts in terms of wave-lengths (750m $\mu$ , 690m $\mu$ , 610m $\mu$ , 570m $\mu$ , 530m $\mu$ , 500m $\mu$ , 460m $\mu$  and 430m $\mu$ ). To make the values obtained with the different spectral filters comparable, corrections are made by comparing each filter in turn with one (430m $\mu$ ) taken as the standard. The transmission value should be multiplied by the correction factor (obtained as above) for the filter used.

<sup>1</sup> If  $D$  = diagonal of the square aperture ( $O_1$ ,  $O_2$ ),  $d$  = the variable diagonal between 0 and  $D$ ,  $a$  = value of the angle between 0 and 360° through which  $W$  turns, and  $i$  = intensity of the light (the area of the aperture) then

$$(1) \ i = \left( \frac{d}{\sqrt{2}} \right)^2 = \left( \frac{a}{360^\circ} \right)^2 \text{ for } d \text{ from 0 to } D, \text{ for } i \text{ from 0 to 1, and for } a \text{ from 0 to } 360^\circ, \text{ and}$$

$$(2) \ a = \sqrt{i \cdot 360^\circ} = 0 \text{ up to } 360^\circ, \text{ for } i \text{ from 0 to 1.}$$

### Procedure and accuracy

It is first necessary to determine the extinction of a solution of known concentration in order to be able to calculate in a simple manner any desired concentration from the amount of its extinction *in the same region of the spectrum*.

The filters required for the measurement are introduced one on each side ( $S_1$  and  $S_2$ ). The two beams of light are then adjusted to equal intensity as indicated by the null-point. For obtaining this,  $r$  may be used as a fine adjustment. The colored solution of the substance in a known concentration is filled into an absorption vessel ( $V_1$ ) of suitable stratum thickness<sup>2</sup> and brought into the path of one of the beams of light. An absorption vessel of similar stratum thickness is filled with the solvent<sup>3</sup> (treated in exactly the same way as the solution) and brought into the path of the other beam of light. This compensates absorption in the solvent and the loss of light on the sides of the absorption vessels. More light is now absorbed by the solution ( $V_1$ ) than the solvent ( $V_2$ ) and thus the photoelectric currents become unequal. Consequently the bridge is thrown out of balance and this is indicated by the deflection of the galvanometer. The recorder,  $W$ , on the side of the solvent is now slowly turned in, *i.e.*, the transmitted light on this side is diminished until the null-point is again restored. After noting the value, the absorption vessels and the contents are interchanged and a second measurement taken. This compensates variations in the wall thickness, if any, of the absorption vessels. From the average of the readings, the extinction coefficient is extracted. This preliminary experiment gives  $K_1$  and  $C_1$ . The test solution is then estimated as above and the value for  $K_2$  is obtained. Knowing  $C_1$ ,  $K_1$ , and  $K_2$ ,  $C_2$  is easily calculated from the equation.

A brief account of the practical procedure of sugar estimation with the above apparatus following BENEDICT's picric acid method (1) is described in the following paragraphs.

The sugars are extracted from the plant material by the method of DASTUR as followed by PARLJA and SARAN (2). The material is boiled in water for over 30 minutes, and is then thoroughly crushed in a mortar till a very fine paste is obtained. The water in which the leaves are boiled is used in making up the volumes of the pastes. The filtrate which contains the sugars is freed from impurities by adding lead acetate and subsequent deleading. The final filtrate is concentrated on a water bath and again filtered for the last time. Two cc. of this sugar solution are taken in a test-tube marked at 12.5 cc. and 25 cc. To the 25-cc. mark the picrate picric acid reagent is added, left for a few minutes and the whole thing filtered. Eight cc. of the filtrate are poured into a similar test-tube and 1 cc. of 20 per cent. sodium

<sup>2</sup> To be determined according to the experiment.

<sup>3</sup> With clear aqueous solutions this vessel is filled with distilled water.

carbonate solution is added. The test-tube is then plugged and immersed in boiling water for 10 minutes, cooled, and diluted to the mark. A brown color is obtained owing to the presence of the reducing sugars.

An equal quantity of distilled water is then treated in the same manner as the sugar solution.

The measurement is then made as described above. From the reading obtained, the concentration is read off directly from a calibration curve (fig. 3).

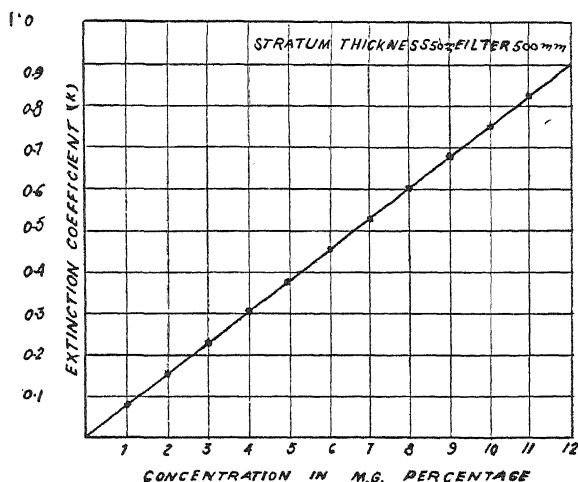


FIG. 3

The determination of  $K$ , and thus also the determination of the concentration, becomes more accurate the greater the absorption of the solution under investigation. Hence accuracy can be increased by increasing the stratum thickness, as can be seen from table I.

TABLE I

ESTIMATION OF A SINGLE SAMPLE OF GLUCOSE (0.4 GRAM PER LITER) USING DIFFERENT STRATUM THICKNESSES

STRATUM THICKNESS	TRANSMISSION	EXTINCTION CO-EFFICIENT (K)	PERCENTAGE ERROR IN CONC. (C)
mm.	%		%
5 .....	90	0.046	± 4.96
10 .....	70	0.155	± 3.35
30 .....	50	0.301	± 2.14
40 .....	30	0.523	± 1.43
50 .....	20	0.699	± 1.22
60 .....	10	1.000	± 1.035
70 .....	5	1.301	± 0.817





## BRIEF PAPERS

### APPLICABILITY OF KUNDT'S RULE TO CHLOROPHYLL

G. MACKINNEY

(WITH ONE FIGURE)

KUNDT'S rule is frequently quoted, either in reference to the state of chlorophyll in the living leaf (3), or in comparing absorption spectra of chlorophyll preparations in different solvents (6). Its use in these respects is usually based on a determination of the wave length of maximal absorption for the main red band. Certain observations (5), indicating an apparent shift in the position of this maximum, may be explained on the basis of different degrees of skewness of the chlorophyll absorption band, and because of the relevance of these observations to KUNDT'S rule, evidence is reviewed, denying the rule either general significance or specific applicability to chlorophyll.

As summarized in 1878 (4), KUNDT'S rule is a statement (Satz): "Man kann mithin als Resultat der ganzen Untersuchung den folgenden Satz aussprechen: Hat ein farbloses Lösungsmittel ein beträchtlich grösseres Brechungs- und Dispersions-vermögen als ein anderes, so liegen die Absorptionsstreifen einer in den Medien gelösten Substanz bei Anwendung des ersten Mittels dem rothem Ende des Spectrums näher als bei Benutzung des Zweiten." HOUSTOUN (2) writes of this (p. 331): "KUNDT in 1874 dissolved substances that produced absorption bands, using different solvents, and found that the position of the band varied with the solvent." HOUSTOUN then quotes KUNDT'S conclusions and he comments: "This statement is not true; the band moves as often the one way as the other, and generally there is a marked change in intensity and shape, in comparison with which the shift in position can be neglected." Discussing anomalous or "selective" dispersion, HOUSTOUN has derived formulae relating the indices of refraction,  $N$  (of the solution),  $n$  (of the solvent), and the absorption coefficient  $k$ , as a function of wave length,  $\lambda$ . If  $\lambda_0$  represent the wave length of free period of vibration of an electron, then absorption will be encountered for the condition  $\lambda \rightarrow \lambda_0$ . It does not seem possible that one may predict, *a priori*, for a hitherto unused solvent of refractive index  $n_1$ , whether  $N$ ,  $\lambda_0$ , or both will be affected, nor whether they will necessarily follow the direction indicated by KUNDT'S rule.

KUNDT'S rule is ordinarily applied to chlorophyll by considering the wave length of maximal absorption as a function of the refractive index of the dissolving medium. In most organic solvents, this maximum lies between 6550 and 6700 Å, while for colloidal chlorophyll, it is close to 6800 Å, and

for chlorophyll *in vivo*, between 6800 and 6820 Å. The curve relating these quantities is then extrapolated to 6800 Å, and on the assumption that KUNDT's rule is obeyed, it is concluded that a medium of abnormally high refractive index would be required, and that therefore chlorophyll cannot be in true solution in the chloroplast. The concomitant requirement of KUNDT, "ein beträchtlich grösseres Dispersions-vermögen," has been largely overlooked, with one notable exception. Thus SORBY (8) also noted that the exact position of the absorption bands of chlorophyll varied considerably according to the solvent, even though inert. "I have carefully determined the order in which various liquids thus raise bands, and compared it with the order of their specific gravity, and of their refractive and dispersive power, but have hitherto failed to recognize any simple connexion between what may be called their *band-raising power* and any other physical property." (Italics as in the original.)

The extensive measurements of BIERMACHER (1) on the pure components of chlorophyll indicate several exceptions to the rule. The writer has also found a specific example of failure, for the blue component, in acetone  $N_D$  1.35886, and in dichloroethane  $N_D$  1.45026. The maxima, determined spectrophotoelectrically, lie at  $6630 \text{ Å} \pm 5 \text{ Å}$ , and the shapes of the two curves are virtually identical over the whole band for these two solvents.

In denying, therefore, a specific applicability of the rule to chlorophyll, we may briefly examine the causes of certain apparent shifts in the position of maximal absorption. When comparisons are made, either in different solvents, or for different preparations, the degree of skewness may introduce an appreciable error in the location of the maximum. The band "axis" determined by visual or photographic methods, may not coincide with the maximum. A study of the absorption for the whole band is therefore desirable, prior to locating the position of the maximum. Visual photometry yields results that are reproducible within quite narrow limits, certainly within  $\pm 15 \text{ Å}$ , for a given observer. SMITH (7) has taken into account the visibility curve in explaining certain discrepancies, between different laboratories. The reasoning may be extended for chlorophyll to show that in the red portion of the spectrum, where within 200 Å, the absorption coefficient for chlorophyll may change by two orders of magnitude, or more, and the visibility function is rapidly approaching zero, the results, however reproducible, may be peculiarly unreliable, particularly in view of the skewness of the band, caused by the introduction of the *b* component, and the superposition of its absorption on that of chlorophyll *a*. This has been indicated (5) in a comparison made between pigments from leaves and from Sultanina grapes, where the visually determined maxima did not agree within 50 Å, because of an illusory displacement of the true maximum. This is further illustrated in figure 1, where two curves are shown, determined

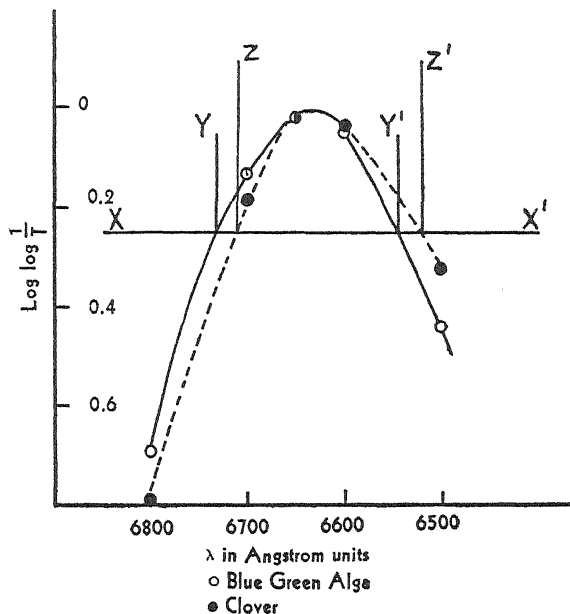


FIG. 1. Curves illustrating the observed band limits of chlorophyll preparations (for explanation see text).

spectrophotoelectrically, both in 80 per cent. aqueous acetone, one for the pigments from a blue green alga, deficient in chlorophyll *b*, the other for clover, considerably richer in this component. If we use a visual instrument and arbitrarily set the photometer at a given transmission level, we may plot an appropriate function of the transmission (in this case, the double logarithm of the reciprocal), as a line, designated  $XX^1$  in the figure, and the observed bands will have as limits the intercepts of the curves with this line, namely  $YY^1$  and  $ZZ^1$ , respectively. As the line  $XX^1$  is moved up the  $Y$  ordinate, the observed difference is minimized but not in practice entirely eliminated.

### Conclusions

The degree of skewness of the absorption curve for chlorophyll solutions (which may be caused by a change either in the dissolving medium or in the ratio of the two components) may introduce an appreciable error in the location of the wave length of maximal absorption. Because of the relevance of these observations to KUNDT's rule, his original statement has been examined. It is of interest that SORBY, contemporary with KUNDT, came to directly opposite conclusions. In justice to KUNDT, no law was formulated.<sup>1</sup>

<sup>1</sup> Although not strictly germane to the subject of this note, KUNDT's attitude is reflected in the following passage (4b): "Der Nachweis, in wie weit die sämtlichen oben

His statement has been found on more searching scrutiny to contain too many exceptions, and receives no theoretical support. With specific reference to chlorophyll, BIERMACHER has noted several exceptions, and the writer has found a similar case with acetone and dichloroethane as solvents.

UNIVERSITY OF CALIFORNIA  
BERKELEY, CALIFORNIA

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dargelegten Anschauungen wirklich in Uebereinstimmung mit der Natur sind, und ihre weitere Ausarbeitung wird die Aufgabe von Specialuntersuchungen sein müssen. Bis diese vorliegen, wird es gut sein noch andere Speculationen, die man allerdings mit Leichtigkeit an das Vorstehende anknüpfen könnte, zu unterlassen.''

## NOTES

**Summer Meeting, Ottawa, Canada.**—The summer meeting at Ottawa, Canada, to be held June 28–30, 1938, in connection with the A.A.A.S. summer meeting, offers many attractive features. Several symposia are being organized, in addition to the general program, and the inspection trips and demonstrations at the Experimental Farm and National Research Laboratories. The suggested symposia topics include *Microelements and Deficiency Diseases*, to be held jointly with phytopathologists; and *Drought Relations*, held jointly with geneticists.

The arrangements are being guided by Dr. G. W. SCARTH, of McGill University, chairman of the program committee for this meeting. Collaborating with him as members of the committee are Mr. B. M. DAVIS, Division of Horticulture, Experiment Farm, Ottawa; Dr. ROBERT NEWTON, National Research Council, Ottawa; Dr. R. D. GIBBS, Department of Botany, McGill University; and Dr. F. P. CULLINAN, *ex officio*. The headquarters have been arranged at the Chateau Laurier, Ottawa. This event provides opportunity for a lively exchange of ideas with our Canadian colleagues, and for a visit to the Canadian seat of government under the most auspicious circumstances. Those who were fortunate enough to attend the meetings at Toronto in 1921, and 1924 (British Association), know the lavish hospitality of Canadian hosts. There should be a large attendance from all sections of North America.

**Richmond Meeting.**—The program committee for the Richmond meeting in December, 1938, is constituted as follows: Dr. W. R. ROBBINS, New Jersey Agricultural Experiment Station, chairman; Dr. H. H. ZIMMERLEY, Virginia Truck Experiment Station, Norfolk, Virginia; and Dr. DANIEL G. CLARK, Cornell University. The secretary-treasurer, Dr. F. P. CULLINAN, is *ex officio* member of the committee. This meeting will be held December 27–29, 1938; those who plan to appear on the programs should make plans early, so that the program committee can organize the papers into suitable groups, and provide commodious rooms for our meetings. Close cooperation with the committee in all matters pertaining to the arrangements is necessary for most effective service.

**Western Section.**—The Western Section of the American Society of Plant Physiologists will meet with the Pacific Division of the A. A. A. S. at San Diego, Tuesday to Friday, June 21–24, 1938. Headquarters will be in Balboa Park, the site of the 1915–1916 and 1935–1936 San Diego Expositions. No hotel has been designated as official. The leading hotels of the city are within 15 minutes walking distance of the grounds.

The tentative program consists of sessions for submitted papers on Tuesday, Thursday, and Friday mornings; a joint symposium with the Western Society of Soil Science on *Salt Tolerance of Plants and Related Problems*, Dr. F. M. EATON, chairman, on Tuesday afternoon; a joint symposium with the section on botanical science on *Plant Invasion on the Pacific Coast* on Wednesday morning; a joint symposium with the Pacific Section of the Botanical Society on *Cell Wall Structure*, Dr. O. L. SPONSLER, chairman, on Wednesday afternoon; and a joint symposium with the Pacific Section of the Botanical Society on a *Résumé of Progress in Plant Science* on Thursday afternoon. A biologists' dinner is scheduled for Wednesday evening, and the present plans call for a plant physiologists' dinner on Thursday evening.

**George James Peirce.**—The American Society of Plant Physiologists, as a mark of its esteem and affection for him, has dedicated this number of PLANT PHYSIOLOGY, in celebration of his seventieth birthday anniversary on March 13, 1938, to Dr. GEORGE JAMES PEIRCE of Stanford University. Dr. PEIRCE was born at Manila, Philippine Islands, son of GEORGE HENRY and LYDIA ELLEN (EATON) PEIRCE. Following his preliminary education, he entered the Lawrence Scientific School of Harvard University from which he received his degree of Bachelor of Science in 1890. Two years later he was awarded a fellowship at Harvard which permitted him to go abroad, and he spent two years, 1892–1894, at Bonn, Munich, and Leipzig. His higher degrees, M.S. and Ph.D., were won at Leipzig in 1894.

During the first two years following his European study, he was assistant professor of botany at Indiana University. In 1897 he married ANNA HOBART of Cincinnati, and went to Stanford University where he found his life work. He was assistant professor of botany there from 1897 to 1900, associate professor to 1910, professor since 1910, and now emeritus professor at Stanford. He has collaborated in various public services, with the United States Forest Service, the Department of Justice, the Palo Alto Board of Health, the Red Cross, and the California Fuel Administration during the World War. He is a member of many societies, both American and foreign, has written several text-books on plant physiology and biology, and has been honored with the presidency of the California Botanical Society (1931), and the Botanical Society of America (1932). The American Society of Plant Physiologists honored him at the Des Moines meeting in 1929 by selecting him as a CHARLES REID BARNES life member of the society. It is a pleasure at this time to extend to him the affectionate and cordial greetings and good wishes of the Society for many more years of activity, with health and strength to enjoy them.

**Errata.**—Additional errors in the first three numbers of volume 12 of PLANT PHYSIOLOGY have been reported to the editor, and they are listed in

the hope that members will make corrections in their copies of the journal. Thanks are due to Dr. W. F. LOEHWING and his students for aid in this connection. In most instances the authors have been consulted, also.

P. 207, line 3, for (8) read (2).

P. 253, line 7 from bottom, for "(11)" read (9).

P. 337, line 5 from bottom, for "obesrve" read observe.

P. 506, line 2, for "(10)" read (9).

P. 529, table I, column 7, item 3, for "1.252" read 0.798.

P. 441, line 9, for "(1)" read (11).

P. 674, line 14, for "definie" read define.

P. 438, reference no. 61, for "ONUNUKI" read OKUNUKI. (This reference deals with auxin b, not auxin a or heteroauxin.)

**Cell Necrobiosis and Protoplasmic Death.**—Dr. W. W. LEPESCHKIN is the author of a monograph entitled *Zell-Nekrobiose und Protoplasma-Tod*, Protoplasma Monographien, zwölfter Band, pp. 198, inclusive of bibliography, published by Gebrüder Borntraeger, Berlin. In this monograph of approximately 145 pages of text LEPESCHKIN brings together for the scientific reader all of the essential evidence which has been presented in the literature on the subject of cell necrobiosis. The clear distinction which he makes between the death of a many-celled organism and the death of its constituent cell units is one which has not been brought emphatically to the attention of biologists before. While it is well known to scientists that the organism may die and its constituent cells go on living for some time afterward, it is scarcely appreciated by what progressive steps death in the individual cell really takes place. Dr. LEPESCHKIN's monograph therefore concerns itself with the progressive steps in the dying of the individual cell, which constitutes cell necrobiosis.

In order to make this clear he discusses in the first part of the monograph and brings to bear thereon the evidence in the literature of the manner in which different agents bring about the death of the cell, as well as the discernible changes which take place in the cytoplasm and in other parts of the cell during the processes of gradual dying. For example, he discusses the morphological changes during cell necrobiosis which take place in living matter, as well as the cytological changes, purely physical changes, chemical changes, and the setting free of energy. In connection with these subjects, LEPESCHKIN discusses his own Vitaid Theory which is well known to those who are conversant with the literature of general physiology.

In the second part of the monograph he discusses special cases of cell necrobiosis and the death of protoplasm. In this he takes up for consideration mechanical influences on living matter, and likewise the influences of extreme heat, extreme cold, desiccation, the influence of radiant energy, and

of acids and bases, as well as of salts and narcotics. The monograph is accompanied by a thoroughgoing bibliography of approximately forty pages and by an author index.

In producing this work Dr. LEPESCHKIN has placed the scientific world much in his debt. With the exemplary clarity and brevity which characterize all of his papers, he gives a picture of his subject which cannot help but be of the greatest interest and importance to every biologist. It is a pleasure to congratulate Dr. LEPESCHKIN on this achievement.—C. B. L.

**Colloid Chemistry.**—During the early part of the year 1935–1936, the George Fisher Baker lectureship in chemistry at Cornell University was held by Dr. R. A. GORTNER of the Agricultural Biochemistry department of the University of Minnesota. The lectures were published in 1937 by the Cornell University Press under the title *Selected topics in colloid chemistry with especial reference to biochemical problems*. The eight topics selected for discussion are as follows: the beginnings of the science; what is colloid chemistry; some basic concepts; some fundamental properties of colloid systems; electrokinetics; surface tension, surface energy, interfacial tension, and molecular orientation; adsorption; and the water relationships of bio-colloids. Having experienced the pleasure of listening to Dr. GORTNER's lectures on colloidal chemistry at Minnesota, the reviewer knows how intensely interesting were these lectures at the time of their delivery. They lose little of their fire and enthusiasm by appearing in printed form. Students will find them delightfully informative and stimulating. They are of greatest value to students of biology who may not always appreciate the pervading importance of the colloidal state in all physiological processes. The book is recommended as part of the required reading in courses in plant physiology. It is priced at \$2.50 per copy, and may be obtained from the Cornell University Press, 124 Roberts Place, Ithaca, New York.

**Determination of Amino Acids.**—The methods of quantitative determination of the individual amino acids have been brought together in a valuable book by Dr. RICHARD J. BLOCK, of the New York State Psychiatric Institute and Hospital. It is published in mimeoprint form with flexible binding by the Burgess Publishing Co., Minneapolis, Minnesota. The methods are presented in eleven chapters, the first five of which deal with groups of acids, as follows: arginine, histidine, and lysine; tyrosine, tryptophane, dihydroxyphenylalanine, and thyroxine; proline, and hydroxyproline; cystine, cysteine, and methionine; and glutamic, aspartic, and hydroxyglutamic acids. The next five chapters deal with single amino acids, alanine, glycine, leucine, phenyl alanine, and serine. The last chapter describes the general methods of separation of the amino acids in mixtures. It includes the meth-



ods of FISCHER, CHERBULIEZ, DAKIN, BRAZIER, and PRZYLECKI and KASPRZYK. These general methods are presented in outline form, diagrams, which should be easily followed. Each chapter closes with a bibliography of the original methods papers, from which the precautions and limitations of the methods must be gleaned by the investigator. The compilation is very helpful, and though plant physiologists have not fractioned their nitrogenous materials to show the individual amino acids involved in metabolism, and do not know the significance of each acid sufficiently to use such information in interpreting metabolic changes, yet we are approaching the necessity of making such detailed studies. To those engaged in studying the hydrolytic products of proteins and protoplasm, this book would be a great time saver. It also contains diagrams of some of the most useful pieces of apparatus needed for the separations. With indexes it contains 85 pages, and is priced at \$3.00 per copy.

**Wheat and Flour Quality.**—A practical work on wheat and flour quality by Dr. C. O. SWANSON of the Kansas State Agricultural College has been published by the Burgess Publishing Co., Minneapolis, Minnesota. It contains 26 chapters, 227 pages, and will be very valuable to the entire milling industry. It is written in simple style, and some of the chapters are of interest to the physiologist, especially chapters VIII to XIV. These bear titles as follows: Whence the substances in wheat; influence of climate and soil on quality; soil nitrogen, the key element in protein content; factors that influence the quantity of protein; enzymes, the tools of life processes; the life activities of the wheat kernel; and germination and diastatic activity. The price of this interesting work is \$4.00 per copy. It comes in mimeoprint with flexible binding, and may be ordered directly from the publishers.

**Fertilizers.**—The second edition of Dr. Firman E. Bear's *Theory and Practice in the Use of Fertilizers* has been published by John Wiley & Sons, Inc., New York. The rapid changes in the manufacture and use of concentrated fertilizers have made a rewriting necessary. In addition, the better knowledge of the function of trace elements in successful plant growth has made the inclusion of a chapter on trace elements in soils and crops necessary. The book is a useful introduction to the theories upon which soil amendment is based, and a practical guide to the user of fertilizers in the control of soil fertility and the improvement of crop yields. The book is quoted at \$4.00 per copy.

**Chemistry of Plant Constituents.**—A summary of the problems of plant chemistry has been prepared by Dr. OLE GISVOLD and Dr. CHARLES H. ROGERS, both of the School of Pharmacy of the University of Minnesota, with the title: *The Chemistry of Plant Constituents*. It is published in mimeo-print form with flexible covers by the Burgess Publishing Co., Minneapolis, Minnesota, at \$3.50 per copy. There are 15 chapters, the introductory chapter dealing with the functions of plant parts, and the inorganic constituents essential to metabolism. The succeeding chapters take up the nature and origin of the following groups of constituents: carbohydrates; vegetable fats; plant and insect waxes; phytosterols; proteins; alkaloids; glycosides; the anthocyanins and anthoxanthins; carotenoids; tannins; terpenes and oxygenated terpenes; vitamins; enzymes; and products of fermentation. Although there is much theoretical speculation involved in the attempt to depict the origin of plant constituents, such speculation is justifiable and necessary as long as definite information as to origins is lacking. There is a great deal of stimulating and valuable information crowded into the 295 pages of text, information for which plant physiologists will be thankful. An index makes browsing for specific information easy. Short bibliographies are used in connection with certain topics, but there is no general bibliography. It should find a hearty welcome among those interested in any phase of plant biochemistry.

# PLANT PHYSIOLOGY

JULY, 1938

## HORMONES AND THE ANALYSIS OF GROWTH<sup>1</sup>

KENNETH V. THIMANN

(WITH ELEVEN FIGURES)

It is now about twenty years since the carefully controlled experiments of PÁAL established finally that growth in plants is under the control of special growth substances or *hormones*. In the intervening period our knowledge has advanced considerably, though mainly only in respect to this one rather well-defined group of hormones, the *auxins*. These substances, as you know, have been isolated from both plant and animal sources, including both seed plants and fungi, and their chemical nature has been thoroughly elucidated. Furthermore, large numbers of synthetic substances have been prepared which have similar actions. Their chemistry will be discussed further below; it is enough for the moment to note that they have rather clearly defined similarities.

The physiology of these growth substances, however, shows much more diversity. The action studied by BOYSEN-JENSEN, PÁAL, SÖDING, WENT, and the other workers up until the last few years was an action upon cell enlargement, causing growth in the simplest sense of the word. The hormone was envisaged as the mechanism by which the influence of the tip of the plant was exerted on the growth of the part below it. However, it was not long before evidence was forthcoming that other quite different influences of one part of the plant upon another were brought about through the action of the same hormones.

The first instance of this was the *inhibition* of one bud by another. It had been previously indicated that this well-defined inhibiting action was perhaps due to a special inhibiting substance, and we were able to show not only that it is, indeed, but also that the substance is identical with auxin. Thus a substance which promotes growth in some tissues also characteristically inhibits it in others.

A more marked instance of the same thing is seen in roots. Here all but

<sup>1</sup> Fifth STEPHEN HALES address, read at the Indianapolis meeting, December 28, 1937.

the most extremely low concentrations of auxins inhibit elongation, so that root tissue, like bud tissue, is readily inhibited by auxin. However, it has recently been found both in our own and other laboratories that *excessively* low concentrations, of the order of 1 gram in 10 million liters, actually accelerate growth of roots. These are concentrations of almost unbelievable lowness. GEIGER has calculated that to dilute a gram of indole-acetic acid so that it is completely inactive on *Zea mays* roots would take an amount of water such that if it had to be brought by rail, it would require 400,000 trains, each of 50 ten-ton wagons. Such trains would comfortably extend around the world, or a quarter of the way to the moon.

This phenomenon of growth acceleration and growth inhibition is probably paralleled in all other plant material. Generally the inhibiting concentrations are much higher than in roots, but the difference is apparently quantitative rather than qualitative. The relation is something like that shown in figure 1.

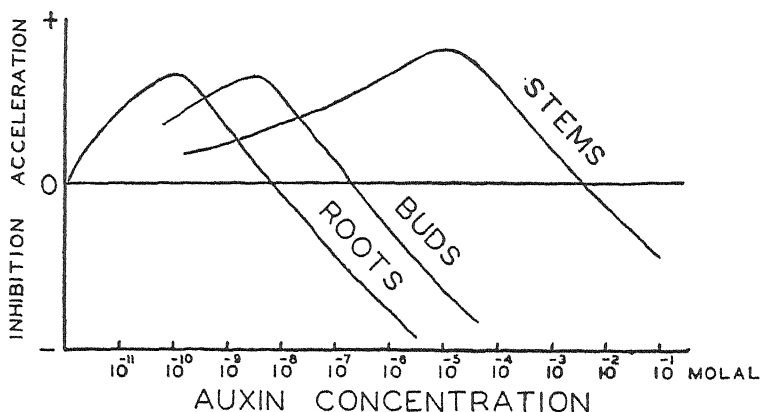


FIG. 1. Scheme for approximate ranges of growth acceleration for different plant organs.

The inhibition of roots involves a very interesting *after-effect* which we have recently been studying, and which is a good example of one type of the analysis of growth. In this case an observed, rather ill-defined acceleration of growth can be traced back to the initial, much slighter acceleration of growth which caused it. If roots are treated with auxin so that they are inhibited, and the auxin afterwards removed, their subsequent growth is accelerated. They catch up with the untreated controls and even surpass them. Figure 2 shows this effect with *Avena* roots. *Triticum* behaves similarly. The higher the concentration of auxin used, the longer it takes for this acceleration to appear, but on the whole the greater the acceleration is when it does come. Thus the roots treated with lowest auxin concentrations are the first to pass the controls, and those treated with highest concen-

trations are the last, but even these plants treated with highest concentrations, whose roots scarcely grow at all during the treatment, afterwards have roots considerably longer than the controls.

At the same time, the very high auxin concentrations also increase the *number* of roots (at least in seedlings of certain plants). In *Avena* the treated seedlings may develop 20 or more roots as against 5 to 7 in controls of the same age (fig. 3). The result of both these responses is that the treated plants, although at first inhibited, subsequently have a far better root system. The roots are both longer and more numerous. As a result the development of the shoot is accelerated, since water supply is exceedingly

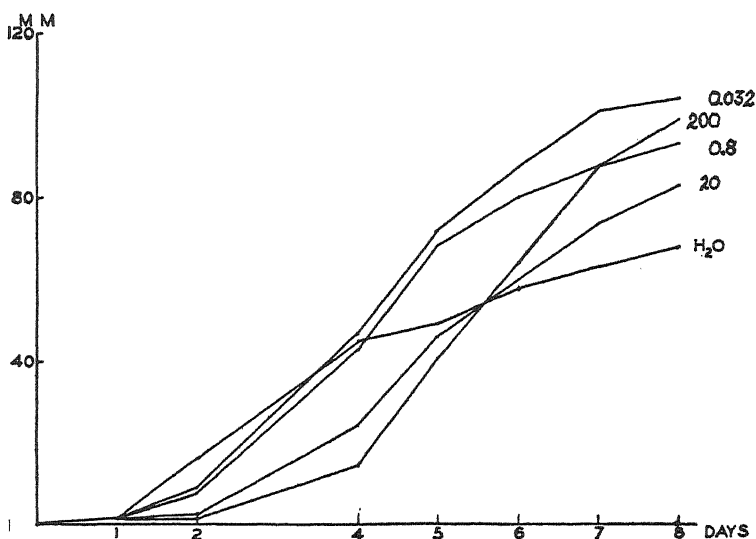


FIG. 2. Length of *Avena* roots, treated with indole-acetic acid at the concentrations marked (in mg. per liter) from the first to the second day of growth and then transferred to water.

important in the growth of young plants. This acceleration of the shoot can be very marked. Figure 4 shows such treated *Avena* plants, after 5 weeks in the cool greenhouse, with controls. Only the plants treated with highest auxin concentration (100 mg. per liter) are shown. The others behaved intermediately. Not only is there a general acceleration, but the leaves are broader and hence the photosynthetic area is increased. This leads to huskier plants. The formation of broader leaves has been obtained in other plant material also.

Thus we have here a growth acceleration resulting weeks later than the initial treatment, involving a number of changes such as increase in height, growth rate and leaf area, which can be analyzed back, with a high degree of probability, to the initial effect on the root system of the seedling. This

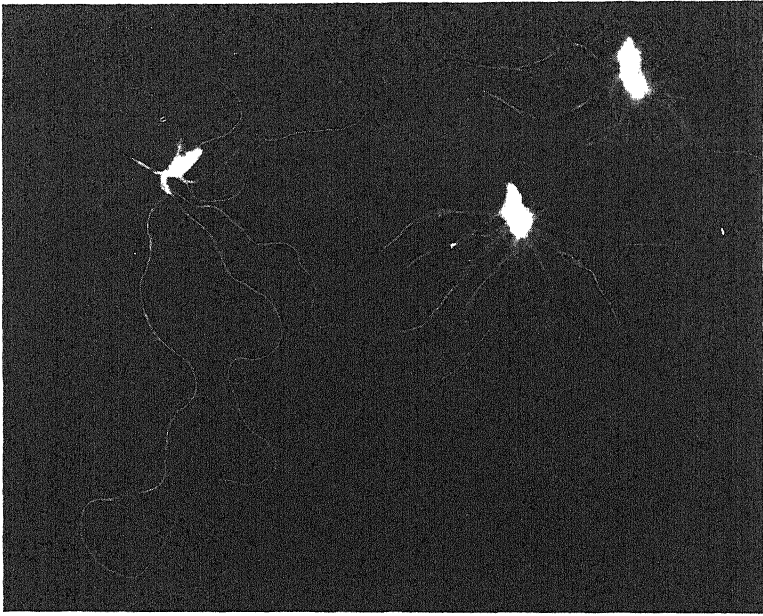


FIG. 3. Right, two *Avena* seedlings treated with indole-acetic acid. Left, control in water.

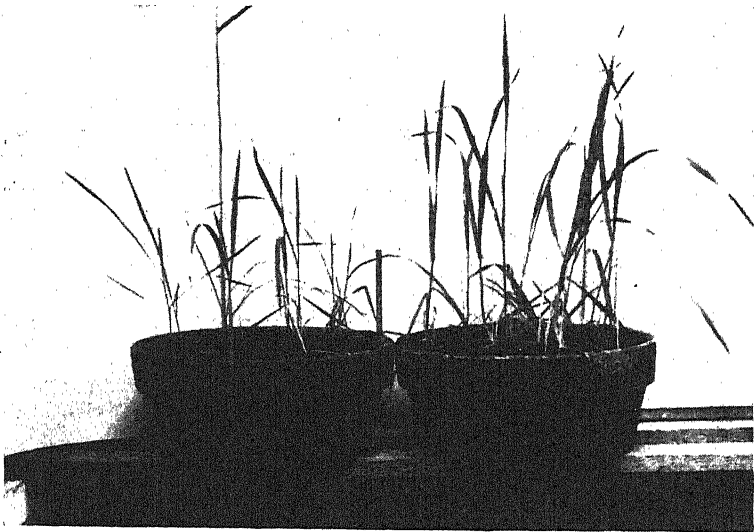


FIG. 4. Right, *Avena* plants grown from seedlings treated during first and second day of growth and transferred to water. Left, controls treated only with water. Five weeks old.

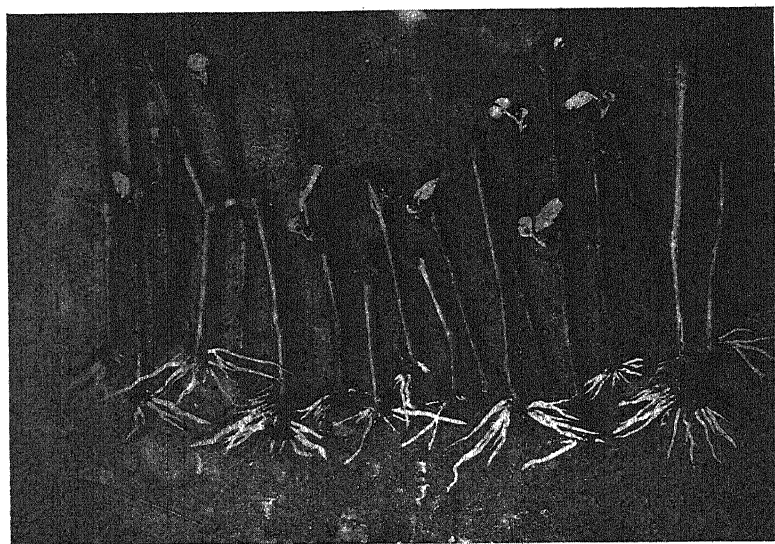
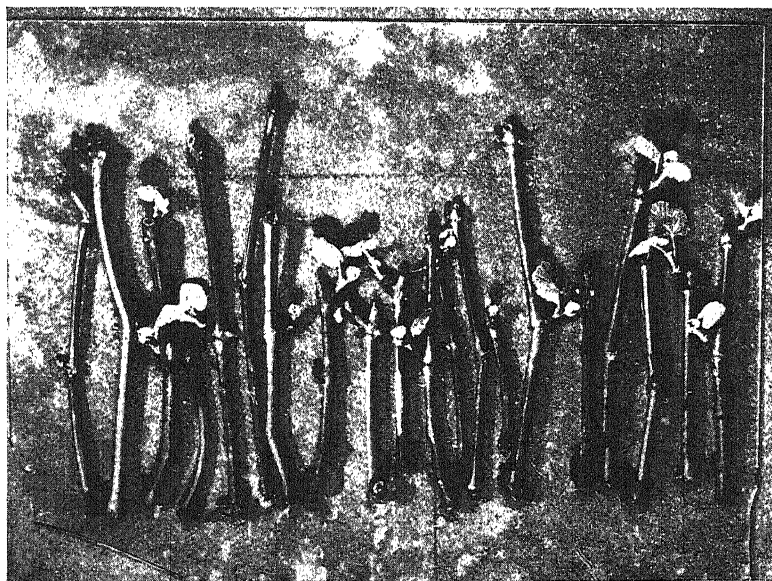


FIG. 5. Concord grape cuttings (one-year wood). Below, treated with indole-acetic acid, 200 mg. per liter for 24 hours at base. Above, controls treated with water. Photographed after 14 days in unheated sand.

shows how complex the analysis of growth phenomena can be, particularly when they are long-term effects.

Now, as you know, another action of the auxins, and it is common, in varying degree, to all the different substances which are auxins, is the induction of root formation. Like other auxin effects this is common to the majority of plants. Figure 5 shows the effect of twenty-four hours' treatment with indoleacetic acid on the rooting of Concord grape cuttings. Many similar photographs have been published recently for a variety of plants. In the normal plant, root-formation is achieved by the migration, to the base, of auxin produced in the growing buds and in the leaves, the transport which obtains normally in the plant being polar. This polarity of the transport of auxin brings about the formation of roots predominantly at the *bases* of cuttings, just as it is also responsible for the influencing of growth by buds or other auxin-forming organs below rather than above them. Of course, if we apply auxin to the plant externally, this polarity of its transport may be obscured by the upward and outward movement to which auxin (like salts or any other externally applied material) is subject. Nevertheless in the intact plant it is evident that there is a rather strict polarity of auxin transport, and the fact that under the right experimental conditions it can be demonstrated and even measured is important for the analysis of growth problems.

The first stages of root development involve rapid cell division and the formation of a root initial. Since this is brought about by auxin, it is perhaps not surprising to find that cell division in other tissues is also activated by auxin. This is mainly true only for the cambium. In tissues other than the cambium, however, cell division can be stimulated, though usually only by unphysiologically high auxin concentrations such as are produced by artificial application, or by the invasion of pathogenic micro-organisms. There does not, therefore, seem to be so clear a distinction between the factors causing division and those causing enlargement as was formerly thought. The influence of auxin in causing the development of parthenocarpic fruits, recently demonstrated by GUSTAFSON, must be considered in the same connection. It is of interest also that the wound hormone, recently studied by BONNER and ENGLISH on the pericarp of the bean, may cause either enlargement alone or enlargement accompanied by cell division according to the variety of bean used as test object. Evidently here also the factors causing the two processes are somewhat interlocked.

We are faced, therefore, with the fact that this one group of hormones, the auxins, bring about a variety of processes of growth and inhibition, and the effect that they exert evidently depends on the kind of cell they enter and on its physiological state. In the earlier days of this work, some four to five years ago, when only the action on cell enlargement was recognized,



it was customary to consider that the auxin causes growth by combining with the cell wall in some way. However, we were able to show that there is no stoichiometrical relation whatever between the auxin that enters the cell and any of the constituents of the cell walls. Further, this variety of physiological functions obviously indicates that the auxin action is exerted upon some very fundamental process in the cell, some kind of "master reaction." The action has been aptly compared to the unlocking of a door; once unlocked, the doorway no longer controls who or what shall pass in or out; these depend upon the conditions on either side of the door. However, the problem of the unlocking of the door becomes more interesting and important than ever because so fundamental. Similar problems of physiology are associated with the action of all hormones and vitamins, and in no case is there a really clear understanding of their actual mode of action.

There have been two main lines of approach to this unlocking problem; one consists in the analysis of the *key*, i.e., the determination of just what properties the hormones must have in order to act—the other is the analysis of the *lock*, i.e., an attempt to determine more nearly the nature of the master reaction and the other processes brought about by auxin. I will discuss each of these briefly.

Let us consider first the key. The formulae of figure 6 show that the

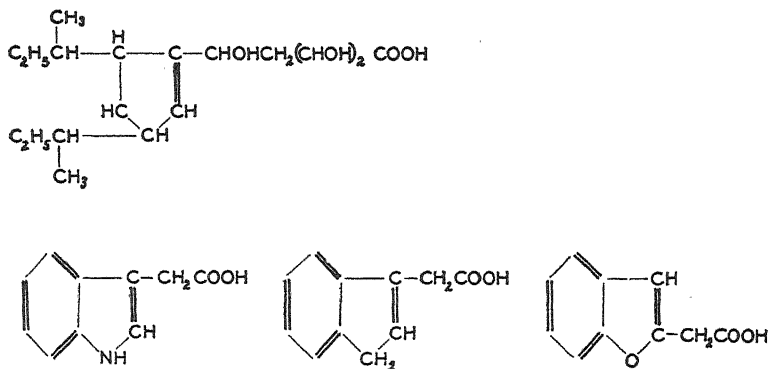


FIG. 6. Above, auxentriolic acid (auxin a) according to KÖGL, HAAGENS MIT, and ERXLÉBEN. Below, left to right, indole-3-acetic acid, indene-3-acetic acid, benzofuran-2-acetic acid.

substances auxin a and indole-acetic acid, the two most highly active auxins, both have an unsaturated 5-membered ring with an acid group in the side-chain. One has a nitrogen atom, the other not. The first subject for study was, therefore, the activity of comparable compounds without the nitrogen atom. Both these substances (formulae to the right) were found to be active, though much less so than indole-acetic acid. A very interesting difference lay, however, in their behavior in the *Avena* test, in which the auxin

is applied in agar to one side of the coleoptile. In this test, indole-acetic acid produces curvature distributed down the coleoptile for 15 mm. or so, but indene-acetic acid, the compound with carbon instead of nitrogen in the ring, produces only localized curvatures (fig. 7). To produce curvature there

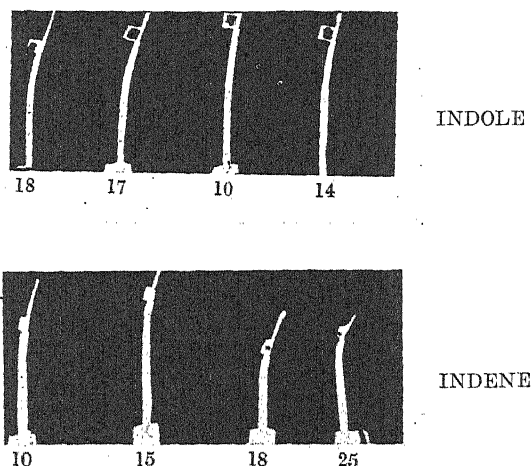


FIG. 7. Decapitated *Avena* seedlings supplied with auxin in agar on one side. Above, indole-3-acetic acid, curvature distributed. Below, indene-3-acetic acid, curvature localized.

must be a marked differential growth and hence the substance must be restricted to the side to which it is applied. If it does not move far on that side it can cause only local curvature—if it spreads out equally on to the other side it can cause no curvature at all. The physiological concentrations of indene-acetic acid evidently do not move as rapidly through the tissues as indole-acetic acid, *i.e.*, their transport is poorer, and this is supported by the fact that indene-acetic acid causes excellent rooting of cuttings if applied at the base, *i.e.*, at the point where its action will occur, but little or no rooting if applied at the tip, from whence it will have to move to the base to bring about its effect.

The second difficulty, that of spreading out, is shown by the oxygen derivative, cumaryl-acetic acid, or benzofurane-acetic acid, which causes straight growth but no curvature. A later analysis of the 3-derivative, benzofurane-3-acetic acid has shown it to be essentially similar though a little more active. Hence here we are dealing with properties which are not essential for *growth* activity, but which control the way in which the growth comes to be manifested; in this case they influence the ability of the substances to be linearly transported.

For further analysis we have therefore tried to study growth activity in

such a way that transport does not enter in. For this purpose immersed plant parts, such as the slit pea stems whose curvatures was shown by WENT to be so convenient a tool for auxin work, have been used. Figure 8 shows

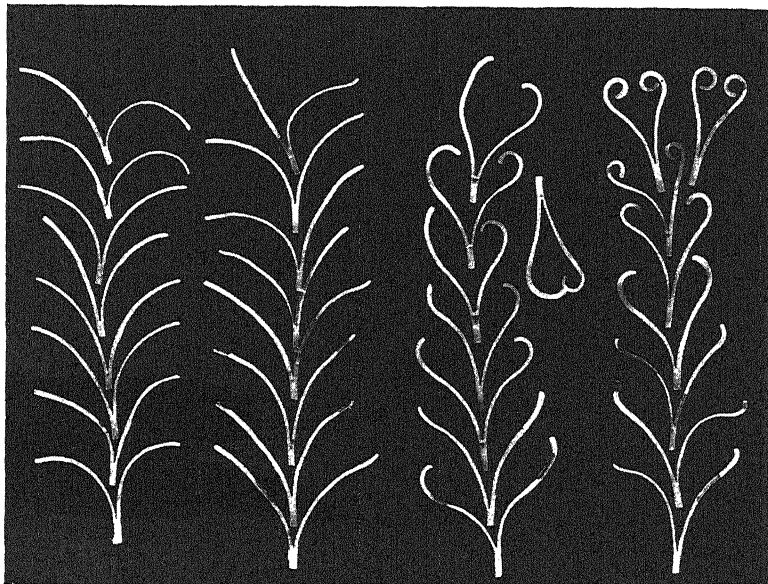


FIG. 8. Curvatures of slit internodes of etiolated pea stems. Left to right, water, 0.1, 1.0 and 4.0 mg. per liter indene-acetic acid.

single young internodes of etiolated *Pisum*, slit longitudinally and immersed in water or auxin solutions. The stem halves, which curve slightly outwards in water, curve inwards in auxin solution on account of differential response to auxin. The inward curvature varies roughly with the logarithm of the auxin concentration. Being immersed, the auxin is applied all round and transport difficulties need not concern us, except insofar as the rates of entry might be different at the different surfaces. One or two interesting points as to the relation between structure and activity have been brought out in this work. First as to the need for the double bond in the auxin molecule. KÖGL found that the saturation of the double bond in indole-acetic acid and auxin caused complete loss of activity (fig. 9). The same thing is shown by phenyl-acetic acid (active) and cyclohexyl-acetic acid (inactive). Cyclohexene-acetic acid, with one double bond, on the other hand, is active. If the double bond is in the sidechain and not in the ring, as in cyclohexylidene-acetic acid, the activity is again lost, hence not only must the double bond be present, but it must be in the ring. Up to now all open-chain compounds have been found inactive.

Secondly as to the structural arrangement (fig. 10). The acid group cannot be next to the ring, since indole-carboxylic and benzoic acids are inactive. Apparently it must be free to reach a certain position in space which is about one carbon atom distance from the ring. Introduction of one methyl group in the side chain does not affect this orientation, but the two methyl groups on the same place prevent it from doing so; to a lesser degree it is prevented from reaching this position by being farther out on the chain, for, in general, activity decreases with increasing length of the sidechain, (phenyl-acetic >  $\beta$  phenyl-propionic >  $\gamma$  phenyl-butyric). A special case is

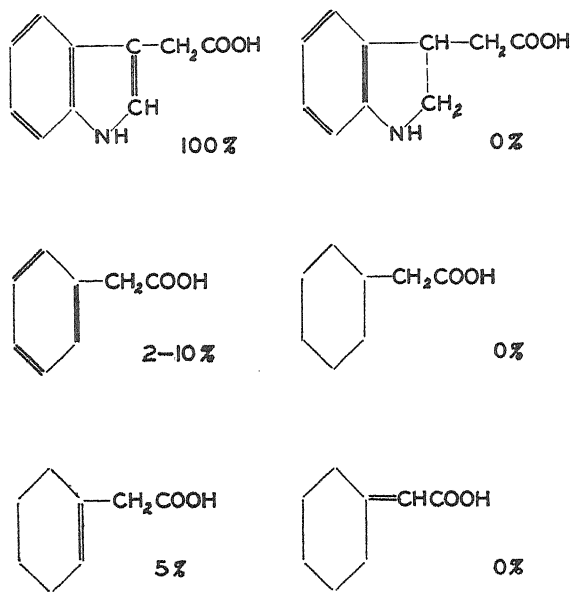


FIG. 9. Top, left, indole-acetic acid; right, dihydro-indole-acetic acid. Middle, left, phenyl-acetic acid; right, cyclohexane-acetic acid. Bottom, left, cyclohexene-acetic acid; right, cyclohexylidene-acetic acid.

that in which the double bond in the sidechain prevents free rotation; *cis*-cinnamic, in which the approximate position can be reached, is active, while *trans*-cinnamic, in which the  $COOH$  is held out at arm's length, so to say, has no activity. The same holds for derivatives of *cis* and *trans*-cinnamic acids. It is evident that within this group of substances the requirements for activity are quite strict, and the action is therefore rather *specific*. When this is more clearly understood we shall be in a position to formulate possible reactions in which the auxin may take part and begin to find out which one occurs.

The other approach is by analysis of the lock. In this, an important piece of evidence is the fact that auxin acts in the tissues together with other sub-

stances. Of these, the best understood is sugar. In order for auxin to cause growth in the coleoptiles, sugar is essential. If the sugar supply is first exhausted from the plant, auxin can cause no growth. If auxin alone is supplied, sugar greatly increases the growth. Not only sugar, however, but other factors are also concerned. This is particularly true in root formation

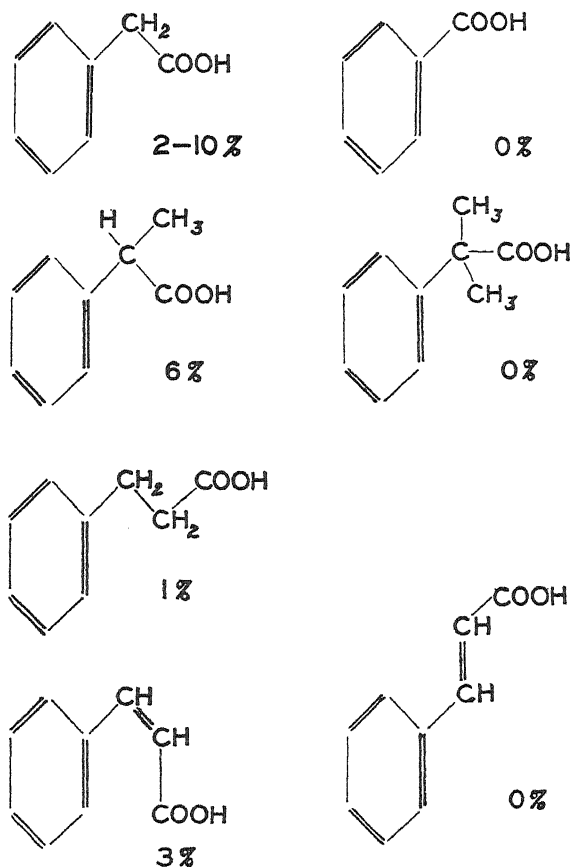


FIG. 10. Top, left, phenyl-acetic acid; right, benzoic acid. 2nd row left,  $\alpha$ -methyl- $\alpha$ -toluic acid; right,  $\alpha\alpha$ -dimethyl- $\alpha$ -toluic acid. 3rd row,  $\beta$ -phenyl-propionic acid. 4th row, left, *Cis*-cinnamic acid; right, *trans*-cinnamic acid.

on cuttings (fig. 11). Here, in *Pisum* cuttings, sugar alone makes about two roots per plant, probably using the auxin already there. Holding the sugar constant and optimal, the number of roots increases proportional to the *auxin* concentration, to a maximum of about 10 roots per plant. Then, holding the auxin constant and maximal, we can increase the next factor (biotin, yeast

growth factor) to reach a new maximum. When this is held at the maximum value, there is evidence for a fourth factor.

Thus the analysis of the lock must take into account the other parts of the lock besides the key. WENT has suggested that these other factors, or certain of them, are really the substances which are specific for the different processes, special substances in the sense of SACHS. A better understanding of the rôle of these other factors will depend on further experiments.

Lastly, as to the nature of the first reaction into which the auxin enters. We now have excellent evidence that it is an oxidation process. I will not

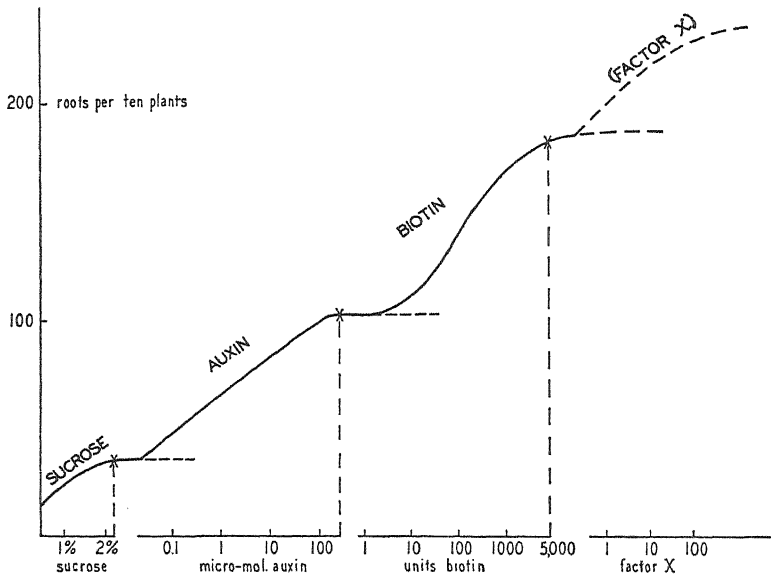


FIG. 11. Interaction of factors causing root formation in *Pisum* cuttings. Ordinate, roots per 10 plants; abscissa, concentration (log. scale) of the factor which is limiting root formation. (From WENT and THIMANN, *Phytohormones*. New York, Macmillan. 1937).

discuss this in detail now, but will only say that when auxin is applied to the cell there is an immediate acceleration of the rate of protoplasmic streaming. This acceleration is observed much sooner than any growth can be detected. It is like growth in that it needs sugar for its effect to be fully realized. It also needs oxygen; in absence of oxygen, streaming is retarded; and in auxin, the consumption of oxygen by this reaction is increased. Hence auxin probably has as its first effect an increase in the rate of one special oxidation process, a process whose substrate is sugar, and which directly controls the rate of streaming, and, probably through this, the rate of growth. The streaming of protoplasm in the cell is something so fundamental that it is easy to see how changes in its rate can bring about a variety of subsequent

changes. It is not yet known whether we can say that this reaction which controls streaming and growth is the primary door which auxin unlocks; if not, it is very near it.

In conclusion I would like to say two things about this analysis of growth problems through hormones. In the first place, it must be remembered that it is plant *physiology*, not *pharmacology*, *i.e.*, we do not, as some people imagine, simply apply all kinds of substances externally to plants, but we are engaged in the study of processes which go on in the normal plant.

Secondly, it is not purely academic. One immediate result of the work has been as you know, the elucidation of the action of auxins in causing root formation on cuttings, a finding of immediate practical utility. In this respect it would have been strongly approved by STEPHEN HALES, who, more than 200 years ago, pointed out that the only sound basis, both of agriculture and horticulture, must be plant physiology; (I quote from his vol. 1): "As the art of Physick has of late years been much improved by a greater knowledge of the animal œconomy, so doubtless a farther insight into the vegetable œconomy must needs proportionably improve our skill in Agriculture and Gardening, which gives me reason to hope that enquiries of this kind will be acceptable to many, who are intent upon improving those innocent, delightful and beneficial Arts: Since they cannot be insensible that the most rational ground for Success in this laudable pursuit must arise from a greater insight into the nature of Plants."

HARVARD BIOLOGICAL LABORATORIES  
CAMBRIDGE, MASSACHUSETTS





# EXANTHEMA IN PEAR, AND COPPER DEFICIENCY

JACOB OSERKOWSKY AND HAROLD E. THOMAS

(WITH FOUR FIGURES)

## Introduction

The present paper deals with a disease of Bartlett pear trees which occurred in two orchards in the coastal region of central California (Contra Costa County). In one orchard the affected trees were scattered, somewhat in groups, over an area of several acres. The majority of these trees were not seriously injured. In the other orchard, several miles distant from the first one, most of the sick trees were severely injured, and occasionally one of them died during the period of observation in 1930 to 1934. The diseased trees were confined in this orchard to an area of about 20 to 25 acres. Insofar as the appearance and growth of the trees was concerned, there was a more or less sharp line of demarcation between the diseased and the healthy areas. Most of the trees in the diseased part of the orchard were suffering from the malady. The various treatments and analytical data concerning the affected trees to be discussed in the following sections refer to trees in the diseased area of this orchard. The disease was diagnosed by SMITH and THOMAS (8) as *exanthema*, and it will be referred to in this paper as such, although this does not imply that the disease is necessarily identical in its causes with other plant disorders designated by the same name.

## DESCRIPTION OF THE DISEASE

The symptoms of the disease are as follows: During the latter part of May or early June the extreme tips of the current-year shoots turn brown and die; almost simultaneously the tips of the terminal shoot leaves also turn brown and die. The dying of the shoots and leaves progresses downwards with the season (fig. 1), and in severely affected trees three-fourths or more of the current year's growth may be dead by the end of the summer. Dead leaves usually fall to the ground leaving the denuded shoots on the tree. This process is repeated year after year and occasionally a tree degenerates to the point of death. As a result of the death of the apical growing points, shoot growth from axillary buds is stimulated, in consequence of which trees affected with *exanthema* present a semi-witches'-broom appearance and become rather dense and bushy (fig. 3). A limited development of bark excrescences results in the bark on older branches becoming somewhat more roughened than on trees unaffected with *exanthema*. The formation of gum has not been observed. Affected trees bear practically no fruit.

Perhaps the most characteristic symptom of *exanthema* in pear is the

appearance of the affected leaves. The dying of the leaves progresses from the tip and the margin toward the center (fig. 2); the dead portion exhibits alternate brown-orange striation running parallel with the outline of the leaf. Not all affected leaves show this striation, but its occurrence in abundance was observed only on pear trees affected with exanthema. In this respect the symptoms of the disease in pear differ from those in other trees (4, 8). Chlorosis as a symptom of exanthema in pear could not be observed



FIG. 1. Pear shoots of the current season exhibiting various degrees of exanthema symptoms. Right, healthy shoot; extreme left, shoot severely affected with exanthema.

by the writers. The green portion of leaves severely "burnt" along the margin may be somewhat faded but the leaves are never typically chlorotic. The symptoms of exanthema are unlike those of any known parasitic disease of the pear.

That the disease is associated with the soil upon which these trees grow can be inferred from the fact that young seedlings of Bartlett pear, the seeds of which were taken from a region free of the disease and grown in that region in pots containing soil from the affected orchard, exhibited, after a few months, symptoms of exanthema.

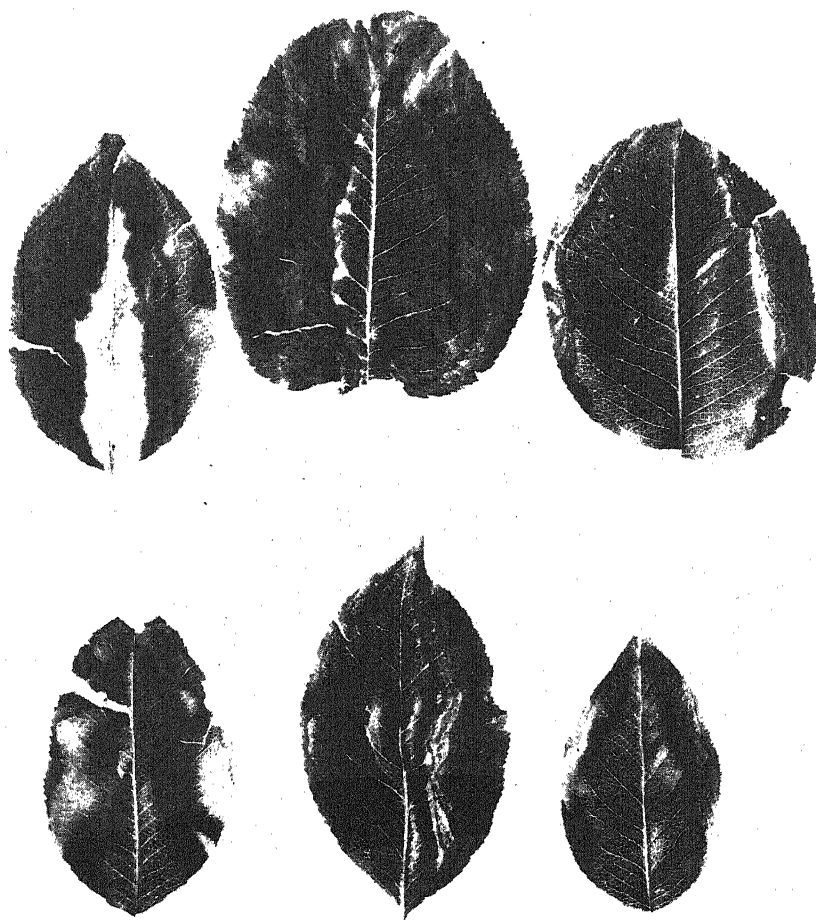


FIG. 2. Pear leaves from exanthema trees, exhibiting tip and marginal burning with striations.

## Experiments

### I. EFFECT OF COPPER ON EXANTHEMA

Copper sulphate crystals were introduced into trees suffering from exanthema, in the lower part of the trunk near the root crown, using the

method described by BENNETT (2) for treatment of chlorotic trees.<sup>1</sup> In order that the quantity of copper sulphate necessary to cure the disease might be determined, the amount applied per tree varied from  $\frac{1}{4}$  to 20 gm. Consideration was also given the size of the tree in determining the amount to apply. These applications were made in February, 1931, before the leaves had started to develop.

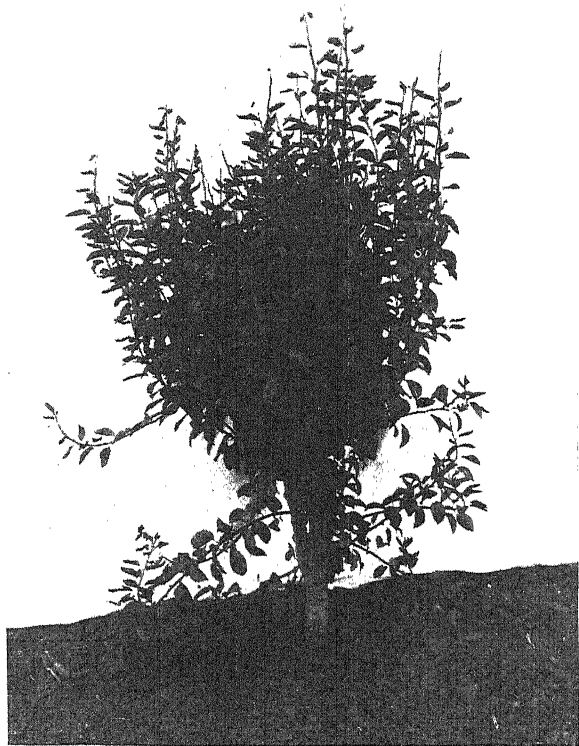


FIG. 3. Pear tree injured by exanthema. Terminal shoots have dead tips and are partially denuded. Numerous leaves are marginally "burnt."

Since the orchard had not been charted for the occurrence of exanthema previous to these treatments, the exact condition of the trees at the time of treatment was somewhat uncertain. In many, however, evidences of the disease were present, even after a severe pruning, and such trees were selected for the application of the copper salts. If the trees had not been

<sup>1</sup> Preliminary accounts of these treatments were published by THOMAS (10) and by OSERKOWSKY and THOMAS (7).

TABLE I  
EFFECT OF INTRODUCTION OF  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  CRYSTALS INTO TRUNK OF PEAR TREES SUFFERING FROM EXANTHEMA

TREATMENT OF TREES	AMOUNT APPLIED	No. OF TREES	CONDITION* OF TREES IN SUMMER															
			1931				1932				1933							
			0	+	++	+++	++++	0	+	++	+++	++++	0	+	++	+++	++++	
IN NUMBER AND PERCENTAGE OF TOTAL TREES IN EACH GROUP																		
Trees treated with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in February, 1931	gm.	3	3	0	0	0	0	0	1	0	0	0	0	2	1	0	0	0
	0.25	4	1	1	0	0	0	0	1	1	0	0	0	0	4	0	0	0
	0.5	7	1	3	1	0	0	0	2	4	1	0	0	1	3	2	1	0
	1.0	11	6	3	0	0	0	0	8	3	0	0	0	7	4	0	0	0
	2.0	3	2	1	0	0	0	0	1	2	0	0	0	3	0	0	0	0
	3.0	5	4	1	0	0	0	0	4	1	0	0	0	3	2	0	0	0
	4.0	2	2	0	0	0	0	0	2	0	0	0	0	2	0	0	0	0
	6.0	2	1	1	0	0	0	0	0	0	0	0	0	2	0	0	0	0
	8.0	4	2	1	0	1	0	0	4	0	0	0	0	4	0	0	0	0
	10.0	2	2	0	0	0	0	0	2	0	0	0	0	2	0	0	0	0
Total no. .... Percentage of total Trees affected with exanthema in 1931 but not treated with copper salts	15.0	2	2	0	0	0	0	0	2	0	0	0	0	2	0	0	0	0
	20.0	2	2	0	0	0	0	0	2	0	0	0	0	2	0	0	0	0
	.....	45	26	11	5	3	0	31	12	2	0	0	0	28	14	2	1	0
	.....	.....	58	24	11	7	0	69	27	4	0	0	0	62	31	4	2	0
	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Total no. ....	0	140	0	12	31	49	48	3	32	36	39	30	6	28	38	47	21	21
Percentage of total	.....	.....	0	9	22	35	34	2	23	26	28	21	4	20	27	34	15	15

\* Symptoms of exanthema: 0 = healthy in appearance, no symptoms; + = slight; ++ = moderate; +++ = moderately severe; ++++ = severe.

treated it is safe to assume that most of them would have exhibited symptoms of exanthema during the summer of 1931. The trees were treated in alternate rows in a block of the orchard severely affected by exanthema. In the summer of 1931 and in each of the two subsequent summers a chart was made of this part of the orchard describing the condition of the trees. The trees were divided into two groups, namely, those treated with copper sulphate and the trees not treated with copper sulphate, which in the summer of 1931 exhibited exanthema. The results are summarized in table I. It is apparent that copper sulphate in small doses cured the trees partially or completely. Further, the beneficial action of copper sulphate lasted over three seasons. The diseased branches on some of the treated trees may be due to an inadequate supply or distribution of copper from the holes in the root crown. Such is also the case in lime-induced chlorosis when the holes containing the iron salt are not properly distributed around the trunk.

Pear trees suffering from exanthema can be cured also by other copper salts. Thus, 7 of 8 diseased trees treated with  $\text{CuCl}_2$  during the dormant season showed very marked improvement during the following growing season. Similar response was obtained with  $\text{Cu}(\text{NO}_3)_2$ . The less water-soluble copper salts also exert a beneficial action on diseased pear trees, although the latter respond more slowly, and one or two seasons may elapse before the action of these salts becomes apparent. This is illustrated in table II. The doses ranged from 1 to 9 gm. of copper salts per tree. The trees were treated in February, 1931.

TABLE II

EFFECT OF INTRODUCTION OF CRYSTALS OF SPARINGLY SOLUBLE COPPER SALTS INTO TRUNK OF PEAR TREES SUFFERING FROM EXANTHEMA. TREES TREATED FEBRUARY, 1931

No. OF TREE	No. OF ROW	TREE TREATED WITH	CONDITION* OF TREES IN SUMMER		
			1931	1932	1933
36	1	Copper carbonate	+++	+	+
38	3	" "	+++	+	tr.
40	4	" "	+++	tr.	0
36	2	Copper phosphate	++	+	+
38	2	" "	tr.	0	0
40	6	" "	+++	+	tr.
36	6	Copper tartrate	++++	++	++
38	5	" "	++	+	0
40	5	" "	+++	+	+

\* Notations same as those of table I. Tr. = trace.

Bordeaux spray also exerts a beneficial action (*cf.*, figs. 3 and 4). Branches on diseased trees sprayed with it early in the spring exhibit vigorous growth and are free of the symptoms of exanthema during the remainder of the season. The action of Bordeaux spray is localized; only the sprayed

branches show recovery from exanthema. The effect of this spray lasts only one season.

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  crystals spread around diseased trees within a radius of a few feet from the trunk and incorporated in the top soil exerted only a slow action on exanthema. Their effect was not apparent until 2 to 3 years after the treatment. Small doses of the copper salt often resulted in slight im-

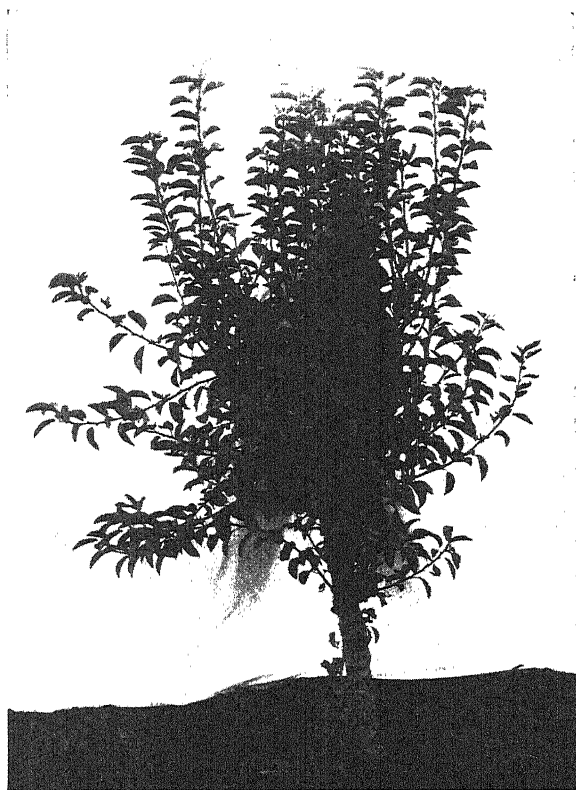


FIG. 4. Pear tree sprayed in the spring with Bordeaux mixture. Originally showed severe exanthema symptoms. Compare with figure 3.

provement only. This is not surprising in view of the fact that the soil in the treated orchard was very heavy, with presumably a large capacity for fixing Cu. Further, the yearly precipitation in the region where the orchard is situated is only about 20 inches. Irrigation is not practiced. As a result of this the  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  dissolves very slowly. Thus, several large crystals of this salt were found on the surface of the soil, and in the uppermost layer of the soil around a treated tree, two years after the application. Table III

TABLE III

EFFECT OF APPLICATION OF CRYSTALS OF  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  TO TOP SOIL AROUND PEAR TREES SUFFERING FROM EXANTHEMA

TREE	Row	DATE OF APPLI- CATION OF $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ TO SOIL	AMOUNT OF $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ APPLIED TO EACH TREE	CONDITION* OF TREES IN SUMMER		
				1931	1932	1933
3	3	February, 1930	<i>lb.</i> 5	++++	+	++
6	3	" "	5	++	+	tr.
8	3	" "	5	+++	tr.	0
7	7	" "	$2\frac{1}{2}$	++	++	+
8	7	" "	$2\frac{1}{2}$	++++	++	+
16	7	February, 1931	50	+++	0	0
16	5	" "	30	+++	++	tr.
16	4	" "	20	+++	+++	+
38	12	" "	20	++++	++	0
38	13	" "	10	++++	+	+
14	5	" "	10	++++	0	tr.
44	12	" "	5	++++	+++	0

\* Notations same as those of table I. Tr. = trace.

presents the results obtained with applications of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  crystals around trees suffering from exanthema.

Affected trees were treated also with salts of various metallic elements. The salt crystals were introduced during the winter into holes bored in the trunk of trees a few inches above the root crown. The method of application was that used by BENNETT (2) for treatment of chlorotic trees. Four or five trees were treated with each salt, and the doses used (equivalent to one or a few grams of the metal per tree) were similar to those of  $\text{CuSO}_4$ , where treatment was beneficial. The following salts were used:  $\text{MnCl}_2$ ,  $\text{ZnSO}_4$ , ferric citrate,  $\text{K}_2\text{Cr}_2(\text{SO}_4)_3$ ,  $\text{NaVO}_3$ ,  $\text{Co}(\text{NO}_3)_2$ , and  $\text{Ni}(\text{NO}_3)_2$ . None of these treatments attenuated exanthema, although in many instances the foliage and twigs were badly injured, indicating that the salts reached the upper parts of the treated trees and were fairly well distributed.

It is thus clear that application of copper salts, whether to the soil in the form of salt crystals, as Bordeaux spray to the leaves, or as crystals introduced into the trunks of trees, resulted in complete recovery of the trees, or at least in a marked reduction in severity of exanthema symptoms and very noticeable increase in growth. Further, copper in small doses (1 to 2 gm. per tree weighing several scores of kilograms) exerted a *specific* beneficial action on trees affected with exanthema, whereas salts of other heavy elements were without action on the disease. This led to the supposition that exanthema may be due to a deficiency of copper. In order to test this hypothesis, analysis of pear tissues from healthy and diseased trees was undertaken. The results of these analyses are discussed in the following sections.



TABLE IV  
COPPER CONTENT OF BARK AND WOOD FROM HEALTHY AND DISEASED PEAR TREES

DESCRIPTION OF SAMPLE	DATE COLLECTED	HEALTHY TREES FROM REGION FREE OF EXANTHEMA		HEALTHY TREES FROM A HEALTHY BLOCK IN AFFECTED ORCHARD		HEALTHY TREES FROM SAME BLOCK AS AFFECTED TREES		AFFECTED TREES		HEALTHY TREES AS RESULT OF COPPER TREATMENT	
		BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD	BARK	WOOD
		Mg. CU PER KG. OF DRY WEIGHT									
Terminal part of shoot .....	{ May 12 May 30 June 1	mg. 12.2 ..... .....	{ 4.9 4.6 ..... .....	mg. ..... ..... 4.5		mg. ..... 3.2 2.7		mg. ..... ..... 14.2			
Basal part of shoot .....	June 1	.....	.....	.....	3.6	4.1	3.5	4.6	16.6	11.6	
Terminal part of shoot .....	July 16	11.8	11.4	6.5	4.4	.....	4.2	2.7	5.5	3.2	
Basal part of shoot .....	July 16	9.0	7.3	7.4	4.0	.....	3.7	1.9	3.7	3.0	
One year or older growth	July 16	12.4	5.9	5.6	2.3	....	3.0	1.2	4.7	1.8	

## II. COPPER CONTENTS OF HEALTHY AND DISEASED TISSUES

Shoots and branches were collected from healthy and diseased trees stripped of their leaves and analyzed for copper. The data are presented in table IV. The samples collected in May were too young to be separated into basal and terminal shoots. In this instance the sample consisted of the upper six inches only, and the bark was not separated from the wood. In subsequent collections the upper six inches of the current-year growth was designated as "terminal part of shoot" while the remaining part, approximately of the same length, was termed "basal part of shoot." Shoots and branches were collected from the affected orchard and from healthy trees in a locality free of the disease (Berkeley). The analytical data show that the copper content was highest in trees grown in the locality free of the disease and in trees in the diseased block which, in consequence of treatment with copper sulphate, recovered from exanthema. The copper content of all samples from trees in the healthy part of the affected orchard was higher than the corresponding values for the samples from the diseased trees. There was, however, no significant difference between the amount of copper in the trees suffering from exanthema and the trees healthy in appearance in the affected part of the orchard. A similar relation holds true also in the case of leaves (table V) and will be discussed below.

Pear leaves were collected from various localities at different times dur-

TABLE V

COPPER CONTENT OF PEAR LEAVES FROM HEALTHY AND DISEASED TREES

GROUP OF SAMPLES	TYPE OF LEAVES	LOCALITY COLLECTED	NO. OF SAMPLES	RANGE OF CU CONTENT IN P.P.M. OF DRY WEIGHT
A	Healthy leaves	Regions free of exanthema	40	<i>p.p.m.</i> 10 -41
B	Healthy leaves	Three regions free of exanthema within $\frac{1}{2}$ to 6 miles from affected orchard	6	12 -17.5
C	Healthy leaves	Healthy block in affected orchard, from 20 to 100 yards from diseased trees	10	4.9- 7.6
D	Healthy leaves	Healthy trees in affected block of orchard	4	4.5- 5.6
E	Healthy leaves from trees suffering from exanthema	Affected block of orchard	11	3.7- 6.7
F	Leaves affected with exanthema	Affected block of orchard	6	3.2- 5.1

ing the growing season and from healthy and diseased trees in the affected orchard and in orchards adjacent to it. The results are given in table V. The *A*-samples were collected from three orchards situated in two regions about 25 and 80 miles, respectively, from the orchard affected with exanthema. Some of the samples are of Hardy pear; the majority, however, are of Bartlett pear trees, the same variety as the affected trees. The inclusion of the Hardy samples in this table has very little effect upon the conclusion to be drawn, since there were no significant differences in copper content between samples of the two varieties. The copper content of the samples from the two localities was also practically identical.

The *B*-samples were obtained from three orchards from  $\frac{1}{2}$  to 6 miles from the affected orchard.

The *C*-samples were gathered from trees healthy in appearance, and of normal vigor, grown in the affected orchard, but on the edge of the affected block. These trees were 20 to 100 yards from the sick trees.

The *D*-samples were obtained from a few trees healthy in appearance scattered in the affected block of the orchard. Although these trees were free of symptoms of exanthema, they made, for the most part, a rather subnormal growth, and thus may be considered as being on the verge of exhibiting exanthema.

The data in table V strikingly show that the copper content of leaves from regions free of exanthema is decidedly higher than that of either healthy or diseased leaves from the affected orchard. The copper content of the former is never below 10 p.p.m. while the highest recorded copper content of the latter was not above 7.6 p.p.m. and even this value is for healthy trees outside the affected portion of the orchard. The average was about 4 p.p.m. There is, however, no significant difference between healthy and diseased leaves from the area affected with exanthema. This fact is not incompatible with the assumption that exanthema is due to copper deficiency. A similar situation is obtained in the case of lime-induced chlorosis curable by administration of iron salts. It was shown in that case that not all of the iron in leaves is active in chlorophyll formation, that only a portion of the iron participates in chlorophyll formation, and this fraction was termed "active iron." Chlorotic leaves are poorer in active iron than green leaves from chlorotic trees, although the iron content of the latter might be below that of the chlorotic leaves (6). Similarly it may be assumed that not all of the copper in leaves is active (whatever that activity may be), and that leaves affected with exanthema contain less active copper than healthy leaves in the affected orchard, although there need be no significant difference in the total copper content of these two kinds of leaves.

## III. COPPER CONTENT OF BASAL AND TERMINAL SHOOT LEAVES

The leaves first to show symptoms of exanthema are the terminal shoot leaves (fig. 1), and the dying of leaves progresses downward. It was expected therefore that the copper content of terminal shoot leaves from sick trees, whether they exhibit symptoms of exanthema or whether they are about to exhibit them (*e.g.*, collected early in the season), would be smaller than that of basal leaves from the same trees. This, however, was not the case, as is readily evident from the data in table VI. Although the terminal leaves, healthy in appearance, are somewhat higher in copper content than the corresponding terminal leaves affected with exanthema, the difference is small and of doubtful significance. In any event the copper content of the affected terminal leaves was *higher* than that of the basal shoot leaves which when collected did not exhibit symptoms of exanthema, although this difference also may not be significant.

The samples collected on July 16, 1931, were obtained at a time of the season when the dying of leaves on exanthema-affected trees had progressed far, while on May 12, 1933, visible injuries to the leaves were just making their appearance. On this date five composite samples of terminal and basal shoots, respectively, were collected (table VI, columns 4, 5). The "healthy

TABLE VI

COPPER CONTENT OF BASAL AND TERMINAL SHOOT LEAVES FROM DISEASED TREES

DESCRIPTION OF LEAVES	COLLECTED 7/16/31		COLLECTED 5/12/33	
	CU CONTENT IN P.P.M. OF DRY WT.	$\frac{\text{Cu}}{\text{N}} \cdot 10^4$	CU CONTENT IN P.P.M. OF DRY WT.	
			SAMPLE 1	SAMPLE 2
Terminal shoot leaves affected with exanthema .....	<i>p.p.m.</i> 4.0	1.6	<i>p.p.m.</i> 4.5	<i>p.p.m.</i> .....
Healthy terminal shoot leaves from trees affected with exanthema .....	4.5	1.9	5.0	6.7
Healthy basal shoot leaves from trees affected with exanthema .....	3.6	1.5	3.5	3.4
Healthy spur leaves from trees affected with exanthema.....	4.2	1.6	.....	.....

terminal shoot leaves" collected on this date from the sick trees may represent leaves on the verge of exhibiting symptoms of exanthema, since it is very likely that if left on the trees for a few weeks longer many of them would have shown the typical symptoms of exanthema. In any event, such terminal

leaves would have begun dying earlier than the basal leaves on the same shoots, and yet the copper content of these terminal leaves was *higher* than that of the basal leaves.

It was thought that the terminal leaves being younger were richer in protoplasm and therefore required for normal functioning larger amounts of copper per unit of dry weight. Since there is no satisfactory measure of the amount of protoplasm in plant tissues, the nitrogen content of the leaves was used as an approximate, although admittedly crude, measure of the protoplasmic content of leaves. The ratio of Cu to N is reported in column 3 of table VI for the samples collected on July 16, 1931. This ratio may be considered as a very rough measure of the ratio of copper to protoplasm. It is seen, however, that the relation thus obtained between healthy and affected terminal and basal shoot leaves is substantially the same as when the copper content is reported on the dry weight basis.

#### IV. COPPER CONTENT OF MARGINAL AND CENTRAL PART OF LEAVES

Since the injury to leaves affected with exanthema manifests itself in progressive dying of the leaves from the tip and margin toward the center, it was reasonable to expect that the central part of the leaf would contain more copper than the marginal and apical part. Composite samples were obtained from sick trees at various times. The margin and tips of the leaves were severed from the central part with scissors. Only one sample consisted of leaves with dead tips. Analysis showed that the central part of this leaf sample contained 2.3 p.p.m. copper, and the dead margin and tip 2.2 p.p.m. This is an insignificant difference. Several more samples of terminal and basal shoot leaves were collected from sick trees, but the leaves of these samples were free of dead margins or tips. If left longer on the trees many of them no doubt would have exhibited symptoms of exanthema. It is permissible therefore to characterize the condition of these leaves as being in the state of incipient exanthema. In this case also, contrary to expectation,

TABLE VII

COMPARISON BETWEEN COPPER CONTENT OF APICAL AND MARGINAL, AND CENTRAL PART OF PEAR LEAVES

DATE OF COLLECTION	TERMINAL SHOOT LEAVES		BASAL SHOOT LEAVES	
	CENTRAL PART OF LEAVES	APICAL AND MARGINAL PART OF LEAVES	CENTRAL PART OF LEAVES	APICAL AND MARGINAL PART OF LEAVES
CU CONTENT IN P.P.M. OF DRY WEIGHT				
	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>
5/31/33 .....	1.7	3.0	1.8	1.7
5/31/33 .....	1.7	2.6	2.0	2.1
6/5/33 .....	.....	.....	1.9	1.9

there was no consistent difference in copper content between the marginal and the central parts of the leaves (table VII).

### Discussion

#### EXANTHEMA AND COPPER DEFICIENCY

Unlike deficiency of iron in higher plants, which, as far as it is known invariably manifests itself as chlorosis, copper deficiency is characterized by a multitude of symptoms. Thus, SOMMER (9) observed that sunflowers, tomatoes, and flax grown in a culture medium deficient in copper made very little growth compared with the check plants adequately supplied with this element. On the other hand, LIPMAN and MACKINNEY (5) found no significant difference in dry weight of barley plants raised in a culture solution deficient in copper, when compared with the check plants. In this instance, however, the barley deficient in copper developed fewer flowers, and produced practically no seeds. Thus in barley, it appears, deficiency of copper resulted in curtailment of the reproductive organs, and hindered normal development of the embryo. HAAS and QUAYLE (4) studied a case of exanthema in citrus which could be cured by copper salts, and which they succeeded in producing by withholding copper from plants grown in pure silica sand and irrigated with a culture solution. This malady must be thus regarded as due to copper deficiency, yet the symptoms are quite different from those recorded by SOMMER, and LIPMAN and MACKINNEY for annual plants. ANDERSSSEN (1) described a chlorosis attributed to a deficiency of copper and affecting some deciduous fruit trees. Pear trees growing in the same region did not exhibit chlorosis but were suffering from a die-back which, as far as could be ascertained from the description of the injury, was similar to the symptom of exanthema on pears. ANDERSSSEN does not mention, however, the striated bands on injured leaves, neither does he specifically refer to the response of affected pear trees to treatment with copper compounds. Sufficient data are lacking to ascertain whether the disorder of pear trees reported by ANDERSSSEN is identical with the exanthema described herein.

It is thus evident that none of the aforementioned descriptions of the various manifestations of injury to higher plants caused by copper deficiency exactly tallies with the symptoms of exanthema on pear. The literature on this subject does not offer, therefore, a criterion for ascertaining whether or not exanthema is due to a copper deficiency. On the other hand, the marked response of pear trees affected with exanthema to various treatments with copper salts in small doses, the specific effect of copper salts, and the low copper content of plant tissues in the affected orchard bespeak in favor of the hypothesis that exanthema in pear trees is due to a copper deficiency. Crucial evidence in support of this view, however, is lacking. The inducement of exanthema in pear seedlings grown in culture solution in the

absence of copper, and its subsequent cure by administration of salts of this metal could be considered as such evidence. It was attempted therefore to grow Bartlett seedlings in a solution free of copper. Unfortunately the plants used in this experiment were injured, probably by excessive amounts of the rarer elements like Pt, Pb, Co, Ni, and Cd which were added to the culture medium.

Since exanthema is produced by some soil factors it might not be illogical to assume that the disorder is brought about by soil microorganisms which either directly attack the roots or else produce harmful substances. Accordingly the effect of copper would be either to kill or inactivate these microorganisms or to neutralize the effect of the toxic compounds produced by them. The first assumption, however, is untenable, since pear shoots on sick trees respond readily to Bordeaux spray, yet in this case curative action of the spray is strictly localized and confined to the sprayed shoots, thus proving that the copper of the spray had not been translocated within the plant. And yet, despite the fact that it did not reach the roots or the root zone it exerted a very beneficial effect. The supposition that exanthema is due to microorganic toxins absorbed by the roots and transferred to the apical parts of the plant seems improbable in view of the specific curative effect of copper and the absence of any beneficial action from other heavy metals like Co, Ni, Cd, Mn, and Zn. There exists the possibility that microorganisms in the soil could interfere with the absorption of copper by the tree roots. For the reasons given and pending evidence to the contrary, the writers prefer to consider exanthema as due to copper deficiency *per se*. This view may account for most of the correlations existing between copper and exanthema, but it does not furnish a simple explanation for the type and progress of the injury as discussed in sections III and IV, unless recourse is had to the hypothesis that terminal leaves and the margin of leaves contain less active copper than basal leaves or the central portion of leaves respectively; or else that the former require larger amounts of the active copper compound for normal functioning.

#### ANALYTICAL DETAILS

All plant material was thoroughly washed in distilled water, and rinsed with it several times prior to drying, excepting two leaf samples obtained in the orchard affected with exanthema. The analytical data obtained with these samples were the same as those secured with non-washed leaves.

Copper was determined colorimetrically essentially according to the method described by ELVEHJEM and LINDOW (3). During the course of the work several minor changes were introduced in the analytical procedure. With the material on hand there was no need to separate iron from copper.

With each set of samples one or two blanks were run, and the copper

values for the samples were corrected for them. The copper content of the samples varied from about 8 to 100 micrograms ( $\mu$ . gm.). It was found that Baer's law holds true with sufficient accuracy for our purpose within at least the following limits of concentration: 14 to 96  $\mu$ . gm. of Cu dissolved in 10 cc. of chloroform as a green pyridine-thiocyanate compound. All determinations, with the exception of a few, were carried out in duplicate or triplicate. The average accuracy of the analytical data is about 5 per cent.; it is somewhat lower in the case of the samples with the smaller copper contents.

### Summary

1. Exanthema on Bartlett pear trees in central California is described. This disease is attributed to soil conditions. Trees suffering from it can be cured by application of copper salts to the soil, by introducing crystals of copper salts into the root crown of the tree, or by spraying affected trees with Bordeaux mixture. Copper salts exert a *specific* beneficial action on trees suffering from exanthema since  $\text{MnCl}_2$ ,  $\text{ZnSO}_4$ , ferric citrate,  $\text{K}_2\text{Cr}_2(\text{SO}_4)_3$ ,  $\text{NaVO}_3$ ,  $\text{CdSO}_4$ ,  $\text{Co}(\text{NO}_3)_2$ , and  $\text{Ni}(\text{NO}_3)_2$  do not correct exanthema.

2. The copper content of leaves and shoots from trees within the area affected with exanthema is invariably lower than that from samples obtained in localities free of the disease. There is, however, no consistent difference in the copper content of healthy and diseased trees in the orchard affected with exanthema. A similar relation exists with regard to iron in chlorotic trees.

3. Exanthema in all probability is due to a deficiency of copper *per se*.

DIVISION OF PLANT PATHOLOGY  
UNIVERSITY OF CALIFORNIA  
BERKELEY, CALIFORNIA

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# WATER RELATIONS IN *BRYOPHYLLUM CALYCINUM* SUBJECTED TO SEVERE DRYING

WALTER BURCHARD WELCH

(WITH TWO FIGURES)

## Introduction

*Bryophyllum calycinum* Salisb. will reproduce vegetatively by means of plantlets formed in the notches of the vegetative leaves. This reproduction, called regeneration by LOEB (9), has been rather thoroughly treated by LOEB (10), REED (17), BRAUN (1), CHILD and BELLAMY (2), and others. A great deal of attention has been paid by these authors to the reproduction of the young plants, but little attention has been given the water relations in the parent leaf during their formation and growth.

In most of the work reported the leaves, when removed from the plant, were placed in contact with water or at least in a moist atmosphere. Under laboratory conditions, without extraneous water, the plantlets appear on the margin of all portions of the leaf without regard to polarity (fig. 1, A, B). The leaves, when hung up by their petioles, produce young plants on the basal half as well as on the apical half, but when placed with one edge in water or in moist soil the shoots will appear only where the leaf is in contact with the water or moist soil.

The parent leaf may dry until it becomes brittle at its central portion, yet the young plants along the margin will still be turgid and continue to increase in size. Since this leaf is capable of living for a long period and reproducing new tissues without additional water being supplied to it, it was proposed that a study be made of the water relations of the parent leaves and their plantlets. A great number of phenomena might be studied in connection with the ability of these leaves to withstand severe drying: osmotic concentration of the juices of the leaves and plantlets, electrical conductivity of these juices, hydration of the ions present in the solutions, relationship of the freezable and unfreezable water to the total water, the thickness of the cuticle, the distribution of stomata, and so on. The work presented in this paper will deal with the freezable and unfreezable water in their relation to the total water, the thickness of the cuticle, and the distribution of stomata.

Unfreezable water has attracted a great deal of attention among plant physiologists who were interested in cold resistance or the ability of plants to become hardened against killing at low temperatures. ROSA (19) found that in cabbage the rate of decrease in the percentage of freezable water during hardening coincided with the rate of hardening of plants against killing by low temperatures. GREATHOUSE and STUART (6) found that

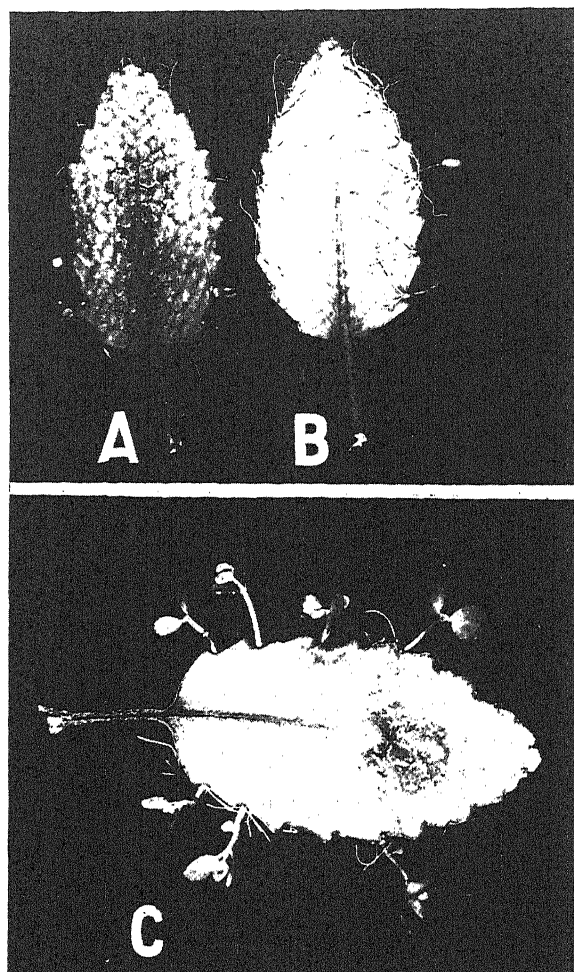


FIG. 1. A. Upper surface of leaf of *Bryophyllum calycinum* after 4 weeks of drying, showing plantlets in notches of leaf ( $\frac{1}{2}$  natural size). B. Lower surface of leaf showing maximum production of roots at 4 weeks ( $\frac{1}{2}$  natural size). C. Upper surface of leaf in A showing plantlets and drying central region after 6 weeks ( $\frac{3}{8}$  natural size).

Ohio and French varieties of red clover could be differentiated as to cold hardiness by the percentage of unfreezable water in the fresh tissues. STARK (21) demonstrated that in general the capacity of an apple twig to retain water against freezing is associated with winter hardiness. He could not, however, grade the hardy and tender varieties in any ascending or descending order on the basis of their ability to retain water in the unfrozen state. In *Pinus rigida* there was only about 5 per cent. more water in the

leaves in the summer than in the winter, and MEYER (13) states that there is no evidence that "bound" water plays any rôle in cold resistance in this species. He suggested "that the basis of cold resistance lies in some as yet not understood, physio-chemical properties of the protoplasm, which probably cannot be discovered by the gross measurements which are generally employed at present." DUNN (3) found that *Bryophyllum*, subjected to a temperature of  $-1.1^{\circ}$  C., would temporarily build up a resistance to this cold temperature to such an extent that a certain number of plants would not be killed after exposure for 15 hours. Surviving plants were propagated for six generations by leaf cuttings. There was a rise in the number of survivors after three generations of cuttings but after six generations there was a return to the original number.

From these and other reports it seems possible that there is a positive correlation between unfreezable water and cold resistance in some species or varieties; but in other species or varieties such a positive correlation between unfreezable water and cold resistance may not be demonstrable.

The terms "free" and "bound" water, used by the earlier investigators, no longer convey the idea investigators wish to express. It is quite possible that under a given set of conditions and at a given time, all or nearly all of the water in a plant may be "bound," and under other conditions and at another time nearly all of the water may be "free." It would be better to refer to the "bound" as unfreezable water and the "free" as freezable water. It then becomes necessary to define the conditions under which the freezing, in the heat of fusion method of determination of freezable water, is carried out. The water is held in the tissues, cells, or parts of cells against freezing in equilibrium with the surrounding atmosphere, or any other tissues, cells, or parts of cells with which they may come in contact. This has been pointed out by GREATHOUSE (5) in his work on red clover roots and tissues of the potato tuber. He found that there was a greater amount of water frozen in the tissues at  $-50^{\circ}$  C. than at  $-22^{\circ}$  C. JONES and GORTNER (7) found that as much water was frozen out of gelatin at  $-6^{\circ}$  C. as was frozen out at  $-50^{\circ}$  C. They suggested that plant tissues should behave as did the gelatin, but the experiences of GREATHOUSE show that there is a freezable-unfreezable water equilibrium that has a different value for every temperature used.

In this work on *Bryophyllum* the temperature of  $-20^{\circ}$  C. was chosen as the one at which the freezable-unfreezable water equilibrium should be measured. There is nothing to suggest that  $-50^{\circ}$  C. would be the lower limit at which the freezable water can be measured; any obtainable temperature above absolute zero might be chosen for such measurements. Since an equilibrium relation is being dealt with, the most important feature is the maintenance of the selected freezing temperature for sufficient time to

permit an equilibrium to become established, and to hold that temperature constant within a range of  $\pm 0.25^{\circ}\text{C}$ .

The problem of drought resistance or of resistance to very severe drying in relation to the unfreezable water content of the tissues has received much less attention than the relation of unfreezable water to cold resistance. VASSILIEV and VASSILIEV (23) found that in general the most conspicuous feature in which a wheat plant, that had been hardened to resist drought, differs from a normal one is in the greater accumulation of hemicelluloses and sugars, chiefly sucrose. The plants which had been exposed to drought by withholding water for 18 days, but were later given an ample water supply, were found to be deficient in water after 8 days of irrigation. They were, however, higher in most forms of carbohydrates except the monosaccharides. This water-deficient, high-carbohydrate condition became more or less "fixed" in plants that had recovered from the drought conditions.

The concentration, osmotic pressure, and "bound" water of the juices of some of the cultivated and some of the wild species of grasses, were found by NEWTON and MARTIN (16) to increase with the progress of maturity. The unfreezable water was found to increase markedly more rapidly than the osmotic pressure and appeared to be more stable. LEBEDINCEV (8) used unfreezable water measurements to determine the water-retaining capacity of xerophytes and mesophytes growing under xerophytic conditions in a semidesert region. She has shown that xerophytes are distinguished by having a higher water-retaining capacity than mesophytes under the same conditions. If the plants were subjected to repeated wiltings there was an increase in the water-retaining capacity which was maintained by the plants after recovery from wilting.

*Bryophyllum* is usually not considered a xerophyte unless MAXIMOV'S (11) criterion, "the main peculiarity of xerophytes is the capacity of enduring permanent wilting without harm or without harm to their subsequent development," is used. The "wilting without harm to their subsequent development" is true only of leaves that are removed from the plant while they are in a turgid condition. If the leaves become dry on the plant and then fall or are removed, they will not produce plantlets on the margin even though roots may have been present at the notches of the leaves while they were still on the plant.

The series of determinations reported in this paper were undertaken to throw light, if possible, on the significance of the unfreezable water in both the parent leaf and the regenerating tissues, and the significance of any shift or redistribution of total water between parent leaf and its plantlets, with special reference to severe drying of the parent leaf and the ability of its regenerating offspring to persist and develop after the parent leaf had partially dried.

### Materials and methods

The *Bryophyllum calycinum* used was that which MEHRLICH (12) has called the Chicago variety. This variety has been grown in the University of Chicago greenhouses for a number of years. Although it has been observed to flower no seeds have been obtained. The plants were vigorously grown under greenhouse conditions until they had produced eight sets of leaves, then the leaves from the fourth and fifth nodes were removed. In preliminary tests, these leaves from the fourth and fifth nodes were found to produce more shoots per leaf than the leaves from any other node. The leaves were allowed to dry in screen-wire trays on a laboratory table for as much as 18 weeks. After a week, the leaf became limp; after 2 weeks, roots appeared at the notches; and after 4 weeks the shoots of the plantlets appeared (fig. 1). The development of the roots and shoots has been adequately described by NAYLOR (15) and YARBROUGH (24). The leaves, after removal from the plant, did not come in contact with any extraneous water except that of the laboratory atmosphere.

The leaf continued to dry until at 10 weeks the central portion, indicated by the shaded area in figure 2, had become tough and brown while the tissues along the edge and base became even more turgid and remained green. The solid line in figure 2 indicates the manner in which the leaf was cut to separate that portion enclosed by the line, hereafter called the central region, from that portion outside, hereafter called the edge and base. This latter portion includes the petiole in all cases.

The method used to determine the unfreezable water was that of RUBNER (20) as modified by ROBINSON (18). Most of the equipment was that described by ROBINSON. The cold chamber was well insulated and deep enough that a minimum change in temperature at the level of the material was observed when the lid was removed. The temperature was maintained at  $-20^{\circ}\text{C.} \pm 0.25$ . Two thermopiles were made of no. 30 gauge double-silk insulated copper and constantan wire, on which two coats of shellac had been brushed. The thermopile used in the cold chamber to check the temperature was one of only five junctions, while the one used in the calorimeter had twenty junctions and produced a potential of 763 microvolts per degree difference in temperature. The "cold" junction in each case was in a thermos bottle filled with a mixture of crushed ice and ice water. The potential in microvolts was read from a Leeds and Northrup type K potentiometer.

The calorimeter was a highly evacuated, highly silvered Dewar flask, 12 mm. in diameter and 65 mm. deep. The flask was sealed into a three-fourths-inch layer of cork with sealing wax at either end, and finally wrapped in tape and varnished. This insulation made it practically impossible to touch the edge of the calorimeter with the fingers during the transfer of the

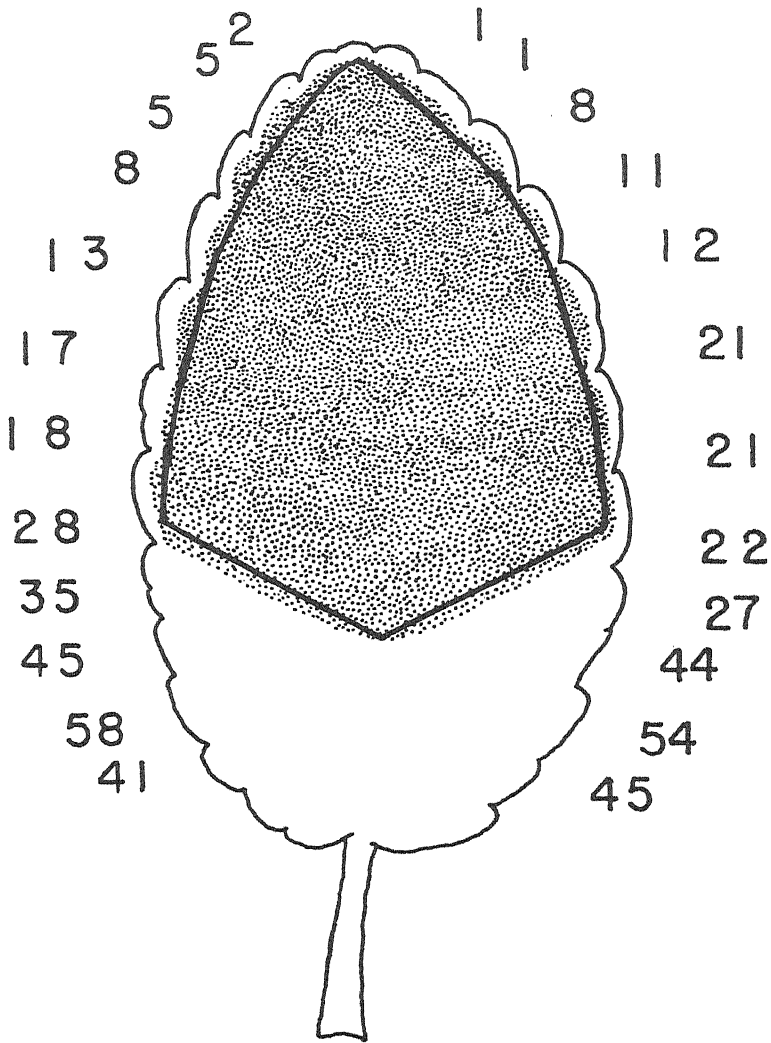


FIG. 2. Shaded portion of leaf of *Bryophyllum calycinum*, which is tough and brown, showing extent of drying at 10 weeks. Solid line shows how leaf was cut to separate central portion from the edge and base. Numbers at margin represent the plantlets counted on 100 leaves varying in age from 8 to 12 weeks.

material from the cold chamber to the calorimeter. The calorimeter was placed in a water bath maintained at 30° C.

A spiral glass stirrer was used to mix the water in the calorimeter. To the stirrer motor a flexible cable was attached which was held in place by two glass bearings in ring stand clamps. The lower end of the cable was attached to a steel bearing which could be fastened rigidly in place. The



glass stirrer fitted into a chuck on the steel bearing shaft. The motor and bearings were so mounted on a hollow rod that the stirrer could be swung in and out of the calorimeter in minimum time; and when not in the calorimeter, the stirrer dipped into a flask of distilled water in the water bath. Owing to the heating of the motor and the bearings, factors from 1.196 to 1.226 had to be used for the thermal capacity of the calorimeter in a continuous series of ten determinations. Never more than ten determinations were made in any one series.

The freezable water was calculated by use of the formula presented by ROBINSON (18):

$$X = \frac{FN(T_2 - T_3) - (WSR + W_1S_1R)}{80 - \frac{T_1}{2}}$$

in which F = correction factor for the thermal capacity of the calorimeter

N = volume of water in cc. used in calorimeter (10 cc.)

T<sub>2</sub> = initial temperature of water in calorimeter

T<sub>3</sub> = final temperature of water in calorimeter

W = total weight of the material

S = specific heat of the material

R = range of temperature between T<sub>1</sub> and T<sub>3</sub>

W<sub>1</sub> = weight of tin-foil cup

S<sub>1</sub> = specific heat of tin foil which is 0.05

T<sub>1</sub> = initial temperature of material in cold chamber

This being below zero appears to have a minus sign but the formula is so constructed that the sign may be disregarded.

X = freezable water

The unfreezable water is found by subtracting the freezable water from the total water present. The specific heat of each specimen was calculated from the specific heat of the dry material, which was found to be 0.0871, and the specific heat of water, 1.

The plant material was chopped with a razor blade and ground to a paste in a mortar. This thoroughly ground material was then placed in a tin-foil cup in a numbered vial and stoppered. The samples ranged in weight from 0.25 to 0.85 gm., but the usual sample weighed about 0.5 gm. After freezing, the cups were removed from the vial and the tops bent down in such a manner as to form waterproof balls, after which they were replaced in the cold chamber and frozen for at least one hour.

The vial was removed from the cold chamber by grasping the stopper with the fingers of the right hand. The upper end of the vial was held with the fingers of the left hand only long enough to remove the stopper and slide the specimen into the calorimeter. After thawing, the material was dried in a vacuum oven at 47° C., using a pressure of 0.04 atm.

## Results

### DETERMINATIONS OF FREEZABLE AND UNFREEZABLE WATER

The data were obtained from three series of experiments. The first series was performed to determine the distribution of the total, freezable, and unfreezable water in the whole plant. This information was necessary not only to establish the normal relationship in the leaves and stem, but also to determine how the leaves at the fourth and fifth nodes compared with the other leaves.

The second series was designed to determine the relationship of total, freezable, and unfreezable water in the whole leaf, with its attached plantlets, during the period of drying. The data from this series were to serve as a guide for the third series of measurements.

In the third series the purpose was to determine the relationship of the water, as in the second series, in the different parts of the leaf and the attached plantlets. In this latter series of measurements it was thought that some light might be thrown on the relation between the freezable and unfreezable water in the parent leaf and in the young plants with special reference to any shift or redistribution of water there may be between the two during the severe drying.

#### SERIES 1. DISTRIBUTION OF TOTAL, FREEZABLE, AND UNFREEZABLE WATER IN THE WHOLE PLANT

The plants used in this series of experiments were vigorously growing individuals that had nine sets of leaves below the apical bud, and a portion of the stem below the ninth set of leaves that was bare. It was impossible to separate the apical bud from the first internode and the first set of leaves and obtain a specimen that would be comparable in weight to the other samples of the stem internodes and the leaves. Therefore the apical bud, first internode, and the first set of leaves were chopped, ground, and frozen as a single sample. The second set of leaves was then removed and treated in the same manner; then the second internode, and so on down the stem.

The lowermost portion of the stem was made up of six very short internodes without leaves, and was treated as a single sample. The determinations on this portion of the stem were entered in table I as the basal internodes. This lower portion of the stem was very woody and rather difficult to reduce to the same degree of fineness as the other samples.

As may be seen from the data of column 2, table I, the first six internodes contained more total water and more freezable water than was found in the leaves attached at the first six nodes, when calculated on the dry weight basis. Internode three contained the largest amount of water of any part examined, and the basal internodes the least. From internode three to internode eight

there was a gradual decrease in total water from 10.385 gm. of water per gram of dry weight to 2.692 gm., and on the same basis the freezable water decreased from 9.706 to 2.368 gm. of water (column 3, table I).

The second and third internodes (column 5, table I), contained a smaller percentage of unfreezable water, 5.62 and 6.53 per cent., respectively, than the leaves at the second and third nodes, 10.40 and 8.66 per cent. The internode contained 7.65 per cent. of unfreezable water while the leaves at the fourth node had only 5.69 per cent. This relation, the higher percentage of unfreezable water in the stem than in the leaves attached to that portion of the stem, was observed in all parts examined, below the fourth node. That portion of the stem referred to as the basal internodes had only 12.05 per cent. of unfreezable water while the seventh internode had 12.66 per cent. This decrease may possibly be due to experimental error, but it may also indicate a reversal of the water relationship in the older stem regions.

The third internode contained more total water per gram of dry weight than any other part of the plant (column 2, table I) and, therefore, less dry weight. There is a smaller percentage of unfreezable water in the third internode than in any of the other internodes. As the stem decreases in total and freezable water, the percentage of unfreezable water increases; or, if the unfreezable water were calculated on a fresh weight basis, it would increase as the dry weight increases. The bud, with the first set of leaves and the first internode, contained more total and freezable water than any of the leaves except the pair at the eighth node. This exception was noted in all plants examined. The eighth pair of leaves appear to be water storage organs. There was a greater percentage, 9.11, of unfreezable water in the bud than in any of the leaves except those at the seventh node which had 9.66 per cent. of unfreezable water. This increased amount of unfreezable water in the leaves at the seventh node is not accounted for, and was seen in all plants examined. The leaves at the fourth and fifth nodes had an average amount of total, freezable, and unfreezable water and were chosen for use in series 2 and 3.

The data in table I are averages of four determinations on three plants where sufficient material was available.

#### SERIES 2. DETERMINATION OF TOTAL, FREEZABLE, AND UNFREEZABLE WATER IN THE WHOLE LEAF

In this series, the whole leaf with its attached plantlets, was treated as a unit, to determine, if possible, the effect of drying on the amount of total, freezable, and unfreezable water. Leaves from the fourth and fifth nodes were placed in screen wire trays and allowed to dry from 1 to 13 weeks. The drying of the leaf was first observable in the central region along the midrib; it then proceeded toward the edge, until at 10 weeks the central

region of the upper portion of the leaf was tough and brown. The edge and base remained turgid and green (fig. 2). The leaves, with attached plantlets, were chopped, ground, and frozen in the manner already described.

TABLE I

TOTAL, FREEZABLE, AND UNFREEZABLE WATER IN LEAVES AND INTERNODES OF *Bryophyllum*  
EXPRESSED IN GRAMS PER GRAM OF DRY WEIGHT, AND THE PERCENTAGE  
UNFREEZABLE WATER

MATERIALS	TOTAL WATER IN GM./GM. DRY WT.	FREEZABLE WATER IN GM./GM. DRY WT.	UNFREEZABLE WATER IN GM./GM. DRY WT.	UNFREEZABLE WATER IN PER CENT.
	gm.	gm.	gm.	%
Bud with first two leaves and internode .....	8.447	7.678	0.769	9.11
2nd leaves .....	7.770	6.691	0.808	10.40
2nd internode .....	10.233	9.657	0.575	5.62
3rd leaves .....	7.355	6.718	0.637	8.66
3rd internode .....	10.385	9.706	0.678	6.53
4th leaves .....	6.470	6.101	0.368	5.69
4th internode .....	9.446	8.723	0.723	7.65
5th leaves .....	5.395	5.018	0.376	6.98
5th internode .....	8.367	7.643	0.723	8.64
6th leaves .....	4.818	4.614	0.204	4.23
6th internode .....	7.189	6.431	0.766	10.64
7th leaves .....	6.409	5.789	0.619	9.66
7th internode .....	3.751	3.276	0.475	12.66
8th leaves .....	9.471	8.776	0.694	7.33
Basal internodes .....	2.692	2.368	0.324	12.05

During the first 2 weeks of drying the leaves became limp and lost 16.54 per cent. of their total water. There was a greater amount of total water, 9.026 gm./gm. dry weight, after 3 weeks of drying than in fresh leaves, 7.719 gm. (column 2, table II). During the third week the roots in the notches of the leaves grew more vigorously than at any other time (fig. 1, B). During the next 8 weeks there was from 18 to 58 per cent. more water in the drying leaf than in the fresh leaf on the dry weight basis. It was during this period that shoots of the regenerating plantlets exhibited the most active growth (fig. 1, C). After 11 weeks the plantlets did not noticeably increase in size.

Since the leaf had no access to water, except that present as vapor in the atmosphere, it is evident that internal changes were causing a reduction in the dry weight. This might be expected, since the energy and materials for the production and growth of the plantlets must be derived from the parent leaf. If there were any increase in internal solids as a result of photosynthetic activity in the leaf or the plantlet, it could not have been measured except as a difference between the material produced and that used by the growing plantlets.

There was a greater percentage of unfreezable water in the leaf and young plants after drying for 13 weeks than in fresh leaves (column 5, table II). There was no gradual increase in the unfreezable water as drying progressed, as might have been expected, but rather a decrease from 8.09 per cent. in the fresh leaf to 3.41 per cent. in the fifth week. The percentage of unfreezable water does not appear to increase or decrease in exact proportion to the total or freezable water, or to the dry weight. It only roughly varies directly with the dry weight, and therefore inversely to the freezable water. Thus any equilibrium that is established between the freezable and unfreezable water must vary from day to day independently of the drying process.

It will be noted that during the eighth week there was less total water than during the seventh and ninth weeks (column 2, table II). The roots had completed their growth during the fifth week and the most vigorous growth of the shoots of the young plants took place during the ninth and tenth weeks. The percentage of unfreezable water in leaves that had dried for 8 weeks was higher than at any time from the second to the thirteenth week (column 5, table II). After 11 weeks the leaves dried out rapidly having less freezable and total water, and in the thirteenth week more unfreezable water in gm./gm. dry weight. This rapid drying is probably the factor that caused the plantlets to stop their growth. They could maintain themselves but could not increase in size on the amount of water they could obtain from the parent leaf.

The data in table II are averages of five determinations on each of three leaves, in all of which, if attached, the plantlets were considered a part of the leaf.

### SERIES 3. DETERMINATION OF TOTAL, FREEZABLE, AND UNFREEZABLE WATER IN PARTS OF THE LEAF AND PLANTLETS, DURING DRYING

In series 3 determinations were made on the different parts of the leaf, and on the plantlets produced at the notches of the leaf to determine the amount of total, freezable, and unfreezable water in each portion and the changes in the quantitative relation to the drying process. Since the leaves first dry out in the central region and leave a margin of green turgid tissue at the edge and base, the leaf was so sectioned that the central region was examined separately from the edge and base. The leaves that had not dried more than 6 weeks were cut along a vein leading to the fourth notch from the base of the leaf; then along the margin, one-quarter of an inch from the edge, to the apex. After 6 weeks, the leaf was limp and yellow (fig. 1, C), in the central region with a definite line of demarcation between the dry area and the turgid area at the edge and base. Then the leaf was cut, as shown in figure 2, along the solid line.

TABLE II

TOTAL, FREEZABLE, AND UNFREEZABLE WATER IN GRAMS PER GRAM OF DRY WEIGHT,  
AND UNFREEZABLE WATER IN PERCENTAGES

MATERIAL	TOTAL WATER IN GM./GM. DRY WT.	FREEZABLE WATER IN GM./GM. DRY WT.	UNFREEZABLE WATER IN GM./GM. DRY WT.	UNFREEZABLE WATER IN PER CENT.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	%
Fresh leaf .....	7.719	7.083	0.625	8.09
1-week-old leaf .....	6.586	6.055	0.530	8.27
2 " " " " .....	6.443	6.019	0.423	6.52
3 " " " " .....	9.026	8.332	0.698	8.05
4 " " " " .....	12.047	10.892	0.655	5.78
5 " " " " .....	12.293	11.890	0.402	3.41
6 " " " " .....	10.745	10.135	0.610	5.81
7 " " " " .....	11.400	10.567	0.833	7.32
8 " " " " .....	9.088	8.372	0.717	7.88
9 " " " " .....	10.420	9.787	0.632	6.07
10 " " " " .....	11.811	11.093	0.718	6.09
11 " " " " .....	11.565	10.737	0.827	7.17
12 " " " " .....	7.802	7.307	0.505	6.44
13 " " " " .....	5.768	5.061	0.707	12.26

Roots appeared after the second week, and shoots after the fourth week (fig. 1, A, B). The most vigorous growth of the shoots took place during the ninth and tenth weeks. After 10 weeks of drying the central portion of the parent leaf was tough and brown, but the edge and base became even more turgid and remained green. After 12 weeks the central region was brittle dry, and after 18 weeks the whole leaf was so dry that it would crack if picked up by the petiole, and the plantlets would fall off unless the leaves were handled with great care.

In all determinations for the first 14 weeks more total water was found in the edge and base than in the central portion of the leaf on the dry weight basis. The percentage of unfreezable water was greater in the central portion than in the tissues at the edge and base during these 14 weeks, except the fifth week, at which time the tissues contained only 7.19 per cent. in the central portion and 9.32 per cent. in the edge and base (columns 4, 7, table III). This exception is not accounted for. In this series the total water decreased during the ninth and tenth weeks as it did during the eighth week in series 2. In the tissues at the edge and base of the leaf in the eleventh week there was more total water per gram of dry weight than at any other time. This parallels the determination in series 2, varying slightly in the time intervals. The total water in the central region of the leaf, on dry weight basis, did not increase after the tenth week of drying but continued to decrease until the leaf was brittle dry. The data taken in the eighteenth week were entered in table III as the central region of the leaf since there was no green or turgid tissue in the edge and base.

TABLE III  
TOTAL AND UNFREEZABLE WATER IN GRAMS PER GRAM DRY WEIGHT AND UNFREEZABLE WATER IN PERCENTAGES

MATERIAL	CENTRAL PORTION OF LEAVES				EDGE AND BASE OF LEAVES				PLANTLETS		
	TOTAL WATER IN GM./GM. DRY WT.	UNFREEZ-ABLE WATER IN GM./GM. DRY WT.	UNFREEZ-ABLE WATER IN PER CENT.	%	TOTAL WATER IN GM./GM. DRY WT.	UNFREEZ-ABLE WATER IN GM./GM. DRY WT.	UNFREEZ-ABLE WATER IN PER CENT.	%	TOTAL WATER IN GM./GM. DRY WT.	UNFREEZ-ABLE WATER IN GM./GM. DRY WT.	UNFREEZ-ABLE WATER IN PER CENT.
Fresh leaf .....	gm.	gm.	%		gm.	gm.	%		gm.	gm.	%
1-week-old leaf ....	5.611	0.839	14.96		6.160	0.664	10.80		.....	.....	.....
2-weeks-old leaf ..	6.004	0.600	10.00		7.135	0.597	8.39		.....	.....	.....
3 " "	5.206	0.523	10.10		5.540	0.519	9.37		.....	.....	.....
4 " "	5.205	0.542	10.41		5.727	0.336	5.39		.....	.....	.....
5 " "	6.187	0.417	6.75		6.466	0.293	4.54		.....	.....	.....
6 " "	6.401	0.459	7.18		7.302	0.680	9.32		5.997	0.696	11.81
7 " "	7.405	0.546	7.37		10.257	0.333	3.27		6.411	0.741	11.56
8 " "	8.295	0.472	5.72		11.473	0.316	2.76		6.256	0.453	7.24
9 " "	7.733	0.384	4.97		11.278	0.423	3.76		6.670	0.402	6.02
10 " "	6.601	0.394	5.97		9.556	0.368	3.80		6.472	0.551	8.52
11 " "	6.748	0.412	6.11		9.179	0.345	3.75		5.962	0.529	8.87
12 " "	6.560	0.835	9.15		13.269	0.895	6.74		6.340	0.597	9.41
13 " "	4.000	0.399	9.98		10.145	0.728	7.19		5.982	0.343	5.73
14 " "	0.170	0.117	68.82		1.451	0.236	18.47		3.675	0.279	7.06
15 " "	0.130	0.063	48.82		.....	.....	.....		4.213	0.427	10.14

The percentage of unfreezable water was greater in the central portion of the leaf in each determination, with the exception of the fifth week, than in the tissues at the edge and base (columns 4, 7, table III).

The first determination on the plantlets was made after the leaves had dried for 5 weeks. The total water per gram of dry weight is less in the young plants from the fifth to tenth weeks than in the central region or the tissues along the edge and base, but after 11 weeks the plantlets had more total water than the central portion but less than was found in the tissues along the edge and base of the leaf. At 18 weeks the plantlets contained about thirty-two times as much total water as the leaf, on the dry weight basis.

The percentage of unfreezable water in the young plants is greater from the fifth to the eleventh weeks than in the parts of the parent leaf examined (columns 4, 7, 10, table III). From the twelfth to the eighteenth weeks there was a smaller percentage of unfreezable water in the plantlets than in the leaf. The greatest percentage of unfreezable water in the plantlets was found during the fifth and sixth weeks.

The data in table III are averages of four determinations on three leaves where material was available.

#### MEASUREMENTS ON CUTICLE THICKNESS AND DISTRIBUTION OF STOMATA ON LEAVES

Since *Bryophyllum* has the ability to retain its water to a remarkable degree, the cuticle and stomata were examined to determine their rôle in this water-retention if possible. The cuticle was examined from prepared slides, and the stomata were counted from impressions made of celloidin peels of the upper and lower surfaces of the leaves.

The cuticle of the upper epidermis was  $4\ \mu$  thick and that of the lower epidermis only  $2\ \mu$ . On the upper epidermis of a mature leaf there were 20 stomata per  $\text{mm.}^2$  and on the lower 46 per  $\text{mm.}^2$ . On the leaf of a plantlet there were 20 stomata per  $\text{mm.}^2$  on the upper and 30 on the lower epidermis. The cuticle is no heavier than on most mesophytes. If the average number on the upper epidermis of a mesophyte is 120 per  $\text{mm.}^2$  and the average for the lower is 140 then the leaves of *Bryophyllum* have fewer stomata than mesophytes. This cuticle and epidermis of the mature leaf and the plantlet could hardly be thought of as responsible for the water-retaining properties of *Bryophyllum*.

TURRELL (22) reports that the ratio of the internally exposed surface to the externally exposed surface of a leaf of *Bryophyllum* is only 7.8. This is the lowest ratio he reported in all of the plants he examined. This feature of the leaf structure may account in part, at least, for the ability of the leaf to hold its water against drying during an 18-week period. The internally



exposed surface, with the reduced number of stomata, may be in part responsible for the water-retaining capacity of the leaf.

### Discussion

The distribution of total water in the whole plant, when the leaves and portions of the stem were examined, indicate that more total water per gram of dry weight was contained in the younger portions of the stem than in the leaves attached to those portions (column 2, table I). There was a higher percentage of unfreezable water in the second and third pairs of leaves than in the second and third internodes of the stem. As the stem became drier in the lower internodes the unfreezable water increased (column 5, table I). The bud, with the first set of leaves and the first internode, contained more total water than any of the leaves except those at the eighth node, which appeared to be water storage organs. The plants used were potted during the late autumn, and the leaves at the eighth node were smaller and thicker, never increasing in size as did the younger leaves.

In these plants in which the whole leaf and stem portions were examined, a large amount of total water was accompanied by a small amount of unfreezable water. The freezable water increased as the total water increased but the unfreezable water decreased, and conversely as the total water decreased the unfreezable water increased. In the leaves this relationship was less striking than in the stem.

When the whole leaf was examined during a period of drying, the total water and freezable water appeared to increase from the third to the eleventh week of drying (columns 2, 3, table II). This apparent increase of water must have been due to an actual decrease in the dry weight, since it is highly improbable that the leaves would take up more water from the atmosphere than they could from a plant growing in well-watered soil.

MOTHES (14) states that there is a greater respiration intensity in wilted leaves than in normal ones. He reported that in *Helianthus* there was a translocation of materials from the older leaves to the younger until the older leaves were exhausted. Any translocation that takes place in the detached leaves of *Bryophyllum* must be from one part of the leaf to another or to the young plantlets on the margin of the leaf. Translocation cannot take place to anything outside of the leaf or the plantlets, and the loss in dry weight must be due to respiration. The greatest loss in dry weight, as measured by the increase of total water in grams per gram dry weight, took place during or immediately following the most vigorous growth of the plantlets at the margin of the leaf. During the period of loss in dry weight the freezable water increased but the unfreezable water decreased.

As the leaf dries, in the manner described in series 2, the unfreezable water decreases until about the fifth week of drying (column 5, table II).

This decrease is not a steady decrease but an irregular one. After the fifth week the percentage of unfreezable water increases, not steadily but irregularly, until at 13 weeks it reaches 12.26 per cent., which is the highest found in the drying leaves. This final percentage is 4.17 per cent. above that of the fresh leaves examined 13 weeks before. The total water per gram of dry weight is 5.768 gm. after 13 weeks of drying as compared with 7.719 gm. in the fresh leaf (column 2, table II). This is one-fourth less total water in the 13-weeks-old leaf than in the fresh leaf on the dry weight basis.

Thus, it might at first appear that there was an increase in the percentage of unfreezable water in the process of severe drying. Almost one-half of the increase took place in the last week. The percentage of unfreezable water was 6.44 per cent. after twelve weeks of drying or 1.65 per cent. less than in the fresh leaf. It was between the determinations made the twelfth and thirteenth weeks that the most rapid drying took place, and the percentage of unfreezable water increased most during the thirteenth week. It therefore appears that the increase in percentage of unfreezable water is not responsible for the capacity of *Bryophyllum* leaves to retain water during severe drying, but rather increased merely as the result of this drying process.

The results of the measurements in series 3 indicate a shift of the total water from the central portion of the leaf to the edge and basal tissues. In the fresh leaf there were 5.611 gm. of total water in the central portion and 6.160 gm. in the edge and base per gram of dry weight (columns 2, 4, table III). At 6 weeks there were 7.405 gm. in the central portion and 10.257 gm. in the edge and base, and at 11 weeks 6.56 gm. in the central region and 13.269 gm. of total water in the edge and base per gram of dry weight. The ratios of total water in the marginal and basal tissues to the central tissues are: in fresh leaves, 1.09; after 6 weeks, 1.37; 11 weeks, 2.02; and at 14 weeks, 8.65. The ratios of grams of unfreezable water in the edge and base to the central region are: in fresh leaves, 0.72; after 6 weeks, 0.43; 11 weeks, 0.74; and 14 weeks, 0.27. Thus the ratio of unfreezable water in the edge and base of the leaf to the central portion decreases markedly as the leaf dries. Since the edge and base resist drying longer than the central region and if the unfreezable water was responsible for this resistance, then the ratios should increase. This was found not to be the case.

The movement of the water from the central region to the edge and basal tissues will maintain the young plants at the margin of the leaves. The increased unfreezable water in the central tissues of the leaf seems to be the result of the shift of the total water to the marginal and basal tissues rather than a means for the shift. The shift may be caused by greater osmotic values of the tissues at the margin. FREELAND (4) found in some of the leaves of *Bryophyllum* that were producing new plants at the margin, a greater osmotic value at the margin than in the central region.

In the plantlets the total water in grams per gram of dry weight was observed to be less than that of the leaf from the fifth to the eleventh week (column 8, table III). However, at the end of 18 weeks there were 4.213 gm. of total water in the plantlets and only 0.130 gm. in the parent leaf on the dry weight basis. The plantlets remained turgid and green while the parent leaf was brittle dry. The percentage of unfreezable water in the plantlets decreased from 11.81 per cent. in the very young plants to 5.73 per cent. after drying for 7 weeks, then increased to 10.14 per cent. after drying for 13 weeks (column 10, table III). If the unfreezable water were responsible for the ability of these plantlets to retain their water it should increase, if not from the first determination then certainly after 13 weeks of drying. This was not the case. In the plantlets there is less relation between the severe drying and the unfreezable water than in the drying leaf.

It appears that the unfreezable water in *Bryophyllum* leaves increases with the severe drying of the leaves and cannot be responsible for the water-retaining capacity of the leaf and plantlets. The unfreezable water shows less correlation to the drying in the plantlets than in the parent leaf.

### Summary

1. The term freezable water is used to designate that state of water referred to by some authors as "free" water, and unfreezable water as that referred to as "bound" water.

2. Since measurements of unfreezable water in the tissues of plants had been frequently employed in attempts to interpret the changes occurring in cold hardening, and very little had been done on drought resistance in relation to the unfreezable water content of tissues it seemed desirable to determine the total, freezable, and unfreezable water in *Bryophyllum* leaves during severe drying.

3. In the whole plant in general there was a correlation between the age of the tissue examined and the percentage of unfreezable water it contained, that is, the older the tissues the greater the percentage of unfreezable water. The bud, however, contained less unfreezable water than did tissues of intermediate age.

4. As the leaf dries out the percentage of unfreezable water increases as a result of the loss of freezable water by evaporation. The increase in the unfreezable water does not seem to the writer to be responsible for the water-retaining ability of the leaf but rather a result of the drying.

5. In the plantlets, where there is the greatest ability to retain the water against atmospheric drying, there is less correlation between the unfreezable water and the drying than in the drying of the parent leaf. The youngest plants on the margin of the leaves have the greatest percentage of unfreezable water.

6. The reduced internally exposed surface and the reduced number of stomata may be responsible for the ability of *Bryophyllum* leaves to retain their water.

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UNIVERSITY OF CHICAGO

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# ASSIMILATION OF AMMONIUM AND NITRATE BY PINEAPPLE PLANTS GROWN IN NUTRIENT SOLUTIONS AND ITS EFFECTS ON NITROGENOUS AND CARBOHYDRATE CONSTITUENTS<sup>1, 2</sup>

C. P. SIDERIS, B. H. KRAUSS, AND H. Y. YOUNG<sup>3</sup>

(WITH TWENTY-TWO FIGURES)

## Introduction

The nutrition of pineapple plants, *Ananas comosus* Merr., with either ammonium or nitrate salts is exceedingly interesting from the point of view of plant growth and performance. Because the complexity of factors involved in a problem of this type is great and a satisfactory solution is difficult without recourse to methods offering greater control of the external nitrogen supply than can be obtained by growing plants in soil, the studies presented in the following pages were carried out with plants grown under greenhouse conditions in complete nutrient solutions containing either ammonium or nitrate nitrogen. In a subsequent publication similar data will be presented on plants grown under various other conditions and fertilized with either ammonium sulphate or sodium nitrate.

STEWART, THOMAS, and HORNER (14) studied the ammonium and nitrate nutrition of pineapple plants with results which may be stated briefly as follows: "The best growth was made by plants growing in the normal nitrate nitrogen cultures; those in one-fourth normal cultures made a very poor growth. Under the conditions of this experiment ammonia nitrogen was not readily utilized from the full strength normal nutrient solution. The plants in the ammonia cultures diluted to one-fourth made a better development than those in the full strength solutions. This would appear to suggest that under field conditions the pineapple plant probably uses nitrogen in both the nitrate and ammoniacal forms." Although the studies of these investigators indicate the direct assimilation of ammonium without previous conversion into nitrate, they have left untouched the phases concerned with the movement and assimilation of ammonium and nitrate nitrogen in the various organs and tissues of the plant.

In 1935 the senior author reported (12) various studies concerned with the relative degree of absorption and movement of nitrate nitrogen and its assimilation by the various sections of pineapple plants. The studies men-

<sup>1</sup> Published with the approval of the Director as technical paper no. 105 of the Pineapple Experiment Station, University of Hawaii.

<sup>2</sup> The second of a series on ammonium and nitrate nutrition.

<sup>3</sup> Presented in part by the senior author in the meetings of the American Society of Plant Physiologists at St. Paul, Minnesota, in June, 1935.

tioned indicated that the assimilation of nitrate in the roots, stem, and white basal leaf tissues was extremely slow. In the green tissues of the leaves the rate of nitrate assimilation was very rapid.

Some of the more outstanding contributions on ammonium and nitrate nutrition have been presented in the first paper of this series (13). For a very comprehensive and thorough review of the literature of nitrogen nutrition the reader is referred to a recent publication by NIGHTINGALE (8).

### Methods of experimentation

#### CULTURAL METHODS

Clon planting material of uniform weight and appearance was employed. The plants were rooted and grown in Mason jars filled with 1800 cc. of nutrient solution containing either ammonium or nitrate as nitrogen sources. The chemical composition of the solution containing ammonium nitrogen is shown in table I.

TABLE I  
COMPOSITION OF AMMONIUM NUTRIENT SOLUTION

SALTS USED	PARTS PER MILLION							
	N	P	K	Ca	Mg	Cl	S	Fe
Ammonium sulphate .....	28						32	
Potassium phosphate .....		15	20					
Potassium sulphate .....			40				32	
Calcium chloride .....				40		70		
Magnesium sulphate .....					24		32	
Iron sulphate .....							3	5
Microelements: B, Zn, Mn, Al, Si, Cu, Ti, Br, I, 0.5 to 1.0 p.p.m. each								
Total .....	28	15	60	40	24	70	99	5

The solution was initially adjusted to pH 5.4 and was maintained at pH  $5.0 \pm 0.6$  by small quantities of 0.1/N NaOH, added at intervals as required.

The composition of the solution containing nitrate nitrogen is shown in table II.

The solution was originally adjusted to pH 4.8 and was maintained at pH  $4.8 \pm 0.6$  with the addition of calculated amounts of 0.1/N  $H_2SO_4$  as required.

The concentrations of the various ions of both nutrient solutions, when compared with those of other well known complete solutions, were very low. The reasons for adopting these low concentrations were (1) the relatively low rate of growth of the pineapple plant; and (2) the comparatively high temperature conditions of the greenhouse during the summer months. Both conditions favor abnormally great accumulation of salts in plant tissues.



TABLE II  
COMPOSITION OF NITRATE NUTRIENT SOLUTION

SALTS USED	PARTS PER MILLION							
	N	P	K	Ca	Mg	Cl	S	Fe
Calcium nitrate .....	28			40				
Potassium phosphate .....		15	20					
Potassium sulphate .....			40				32	
Magnesium sulphate .....					24		32	
Iron sulphate .....							3	5
Microelements: B, Zn, Mn, Al, Si, Cu, Ti, Br, I, 0.5 to 1.0 p.p.m. each								
Total .....	28	15	60	40	24	0	67	5

The nutrient solutions were changed weekly, at which time the volume of absorbed and evaporated water and the weight of the plants were determined. At the time of harvest the plants were about four and a half months old.

#### METHODS OF SAMPLING

For the preparation of the plant tissues for chemical analysis the leaves were detached from the stem and divided, on the basis of maturity, into four groups. These groups were further subdivided into sections of different age and physiological function as indicated.

LEAF AND STEM GROUPING AND SECTIONING.—Pineapple plants (fig. 1) are propagated under field conditions from three asexually produced vegetative organs known respectively as suckers, slips, and crowns, each of which re-



A B  
FIG. 1. Pineapple plants (A) before, and (B) after development.

quires a different period from planting to fruiting on account of differences in initial weight and stage of ontogenetic development. The initial number, length, and weight of the leaves and the length, diameter, and weight of the stem of these various types of propagating material vary. The leaves which are produced at successive intervals after planting increase in length, width, and weight until the fruiting stage is reached, as shown in figure 1-B. All leaves formed during the latter stage are comparatively small and are borne on the peduncle. Pineapple plants bear leaves simultaneously with stages of development ranging from embryonic to senescent. Similar differences in developmental stage may be found between the tissues of the basal and terminal sections of the same leaves. The basal sections, on account of their association with meristematic tissues, are from the point of view of anatomical and functional development embryonic, while the terminal sections are senescent.

With these facts under consideration we divide the entire leaf system of the plant, as well as possible, into chronologically homogeneous groups, as shown in figure 2, where 25 per cent. of the representative leaves of each

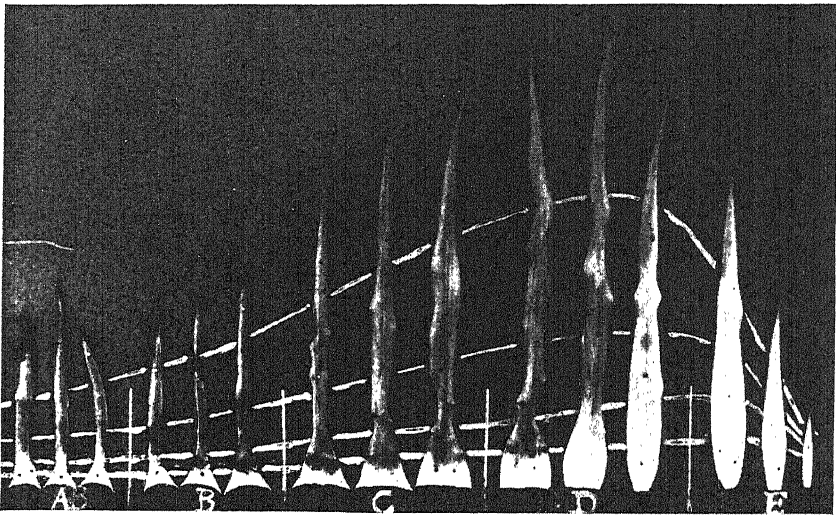


FIG. 2. Different leaf groups, and sections, as indicated with the white lines.

group are shown. Each group was then subdivided into morphologically and physiologically homogeneous sections as shown in figures 2 and 6. The stem was also divided into sections, each one of which corresponded to and was delineated by the region of attachment of the respective leaf groups as shown in figure 4.

The characteristic morphological features of leaves associated with the

chronological development of the different groups of six-months-old plants may be stated as follows:

LEAF GROUPS.—

- A *Old leaves of the planting material.*—These are the leaves which, after planting, produce no new growth, as attested by the absence of groups of lateral spines at distances slightly above the base (figs. 1-a, 2, 6).
- B *Leaves of the planting material with new growth.*—They represent

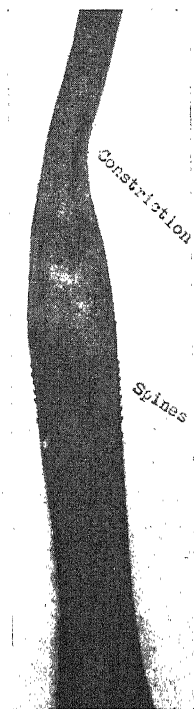


FIG. 3. A leaf of the B group showing spines and constrictions.

leaves already present on the planting material and which after planting produce new growth as attested by the lateral spines and the point of constriction of the leaf (figs. 1, 2, 3, 6).

- C *Mature leaves produced after planting.*—The most mature of the new leaves. They have broad bases containing little or no succulent-brittle non-chlorophyllous tissue and have lateral points at the base decidedly expanded.<sup>4</sup> Their number varies from 6 to 15 according to plant age (figs. 1, 2, 5, 6).

<sup>4</sup> "Expanded" indicates stretched condition.

- D *Active or longest leaves.*—The longest leaves on the plant number from 6 to 12, depending on plant-size and age. In non-fruit-bearing plants, the leaf-base consists of succulent-brittle non-chlorophyllous tissues, slightly broader than the green blade proper, and with non-expanded lateral points; while in fruit-bearing plants it lacks succulent-brittle non-chlorophyllous tissues, is tough and with lateral points prominently expanded (figs. 1-a, 2, 5, 6).

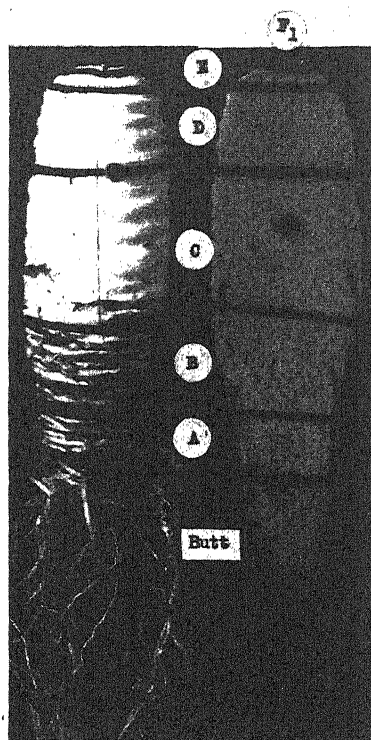


FIG. 4. External (left) and internal (right) view of a stem.

- E *Immature or shoulder leaves.*—These are borne on the shoulder of the stem (transition zone from stem proper to peduncle) and may number from 6 to 12, depending on plant age and size. In non-fruit-bearing plants the base of the leaves is narrower than the green blade proper and contains great amounts of succulent-brittle non-chlorophyllous tissues. In fruit-bearing plants these leaves occupy the region between the suckers and the base of the peduncle. Then their bases become broader than the green blade proper, and lack succulent-brittle non-chlorophyllous tissues (figs. 1, 2, 6).

F<sub>1</sub> *Youngest or apical leaves*.—The youngest leaves on the plant in stages preceding peduncle formation. They may number from 9 to 18, depending on the size and age of the plant. The width of their base is either the same or slightly narrower than that of the green blade proper (figs. 1, 2, 6).

The nomenclature adopted for the different leaf sections is based on certain morphological characters and on chlorophyll distribution. They are shown in figure 5 and are as follows:

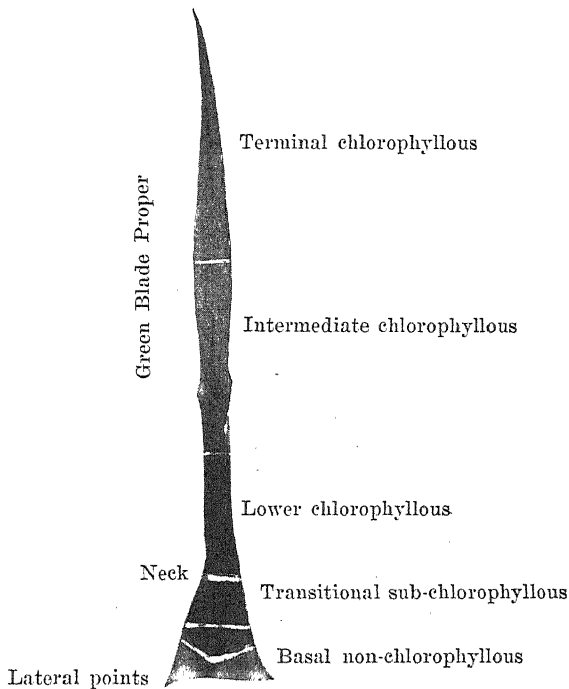


FIG. 5. Nomenclature of the different sections and other parts of a leaf.

#### LEAF SECTIONS.—

- 1 *Basal-non-chlorophyllous*.—The white basal tissues of leaves. (It varies considerably in area and weight in the respective groups of leaves according to their stage of development.)
- 2 *Transitional sub-chlorophyllous*.—The transition region between the chlorophyll-free base and the chlorophyllous tissues of the green blade proper, better delimited in mature leaves by the neck, and in immature ones by the lower limit of the chlorophyllous tissues.
- 3 *Lower chlorophyllous*.—Approximately the lower third of the green blade proper.

4. *Intermediate chlorophyllous*.—Approximately the middle third of the green blade proper.
5. *Terminal chlorophyllous*.—Approximately the terminal third of the green blade proper.

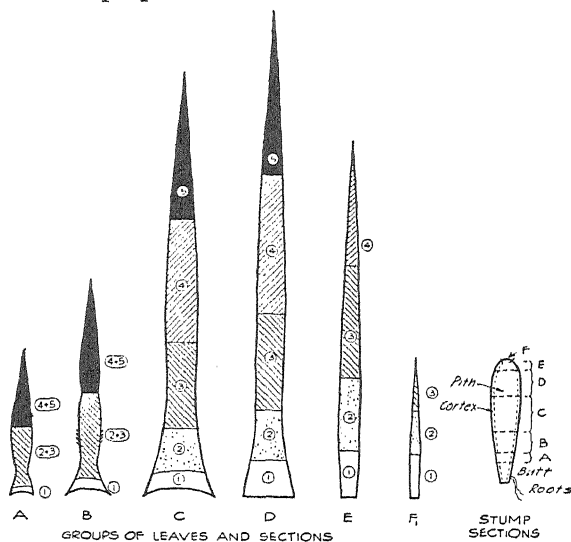


FIG. 6. Diagrammatic representation of the leaves, etc.

The relative amounts of the tissues making up each plant section that was employed for an analytical sample may be obtained by referring to figures 6 and 7 where the different sections are well defined and the magnitude of each section is represented on a percentage basis.

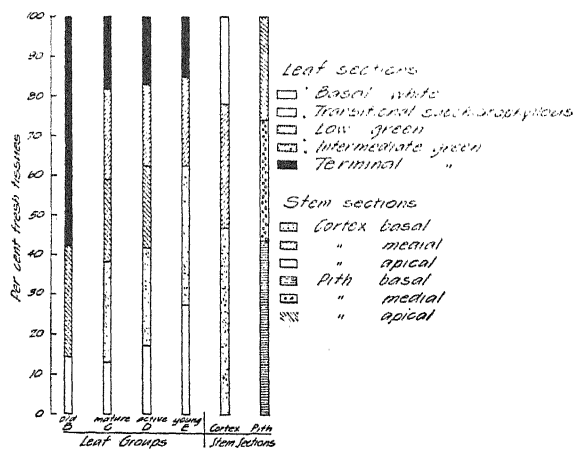


FIG. 7. Relative percentage distribution of the weights of the various leaf and stem sections which were each employed as separate analytical samples.

## ANALYTICAL METHODS

For each sample between 25 and 50 grams of fresh tissues, obtained from each section of the plant as described, were shredded and placed in flasks containing about 100 ml. of boiling water, and kept boiling for a period of 20 to 40 seconds, to inactivate any enzymes present. The flasks were then allowed to cool and toluene was added. Shortly afterwards, or at most in the course of a month, the various samples were ground in a brass mortar with quartz sand and analysed for different fractions of inorganic, soluble organic, and protein nitrogen, and for total and reducing sugars. The methods employed for the analysis of different nitrogenous fractions and sugars have been presented and discussed in the first paper of this series (13). Total sugars were determined after hydrolysis with invertase.

Chlorophyll was extracted from fresh tissues and was separated from the carotenoid pigment according to SCHERTZ's method (9). It was then determined colorimetrically using GUTHRIE's (2) standard.

The water content of the various sections was determined after drying fresh tissues of known weights in a drier at about 100° C.

## Results

## RATE AND AMOUNT OF GROWTH, WATER ABSORPTION, MOISTURE AND CHLOROPHYLL CONTENT

As plotted against time the growth curve of pineapple plants (fig. 8)

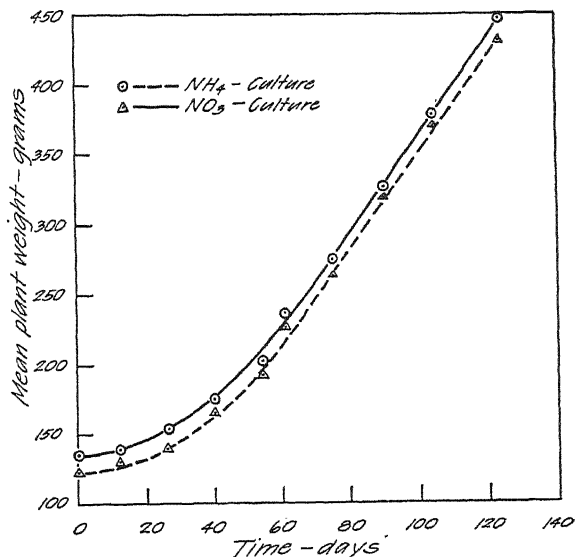


FIG. 8. Relative growth in grams of plants at different stages of development with either ammonium or nitrate nutrition.

five months old is J-shaped, because it represents only about one-half of the growth cycle of the plant. For plants having completed their growth cycle, a sigmoid curve, resembling that of autocatalysis, is obtained. Such a curve was obtained by STEWART, THOMAS, AND HORNER (14) and by HORNER (4) for plants grown under field conditions.

TABLE III

MEAN WEIGHT OF PLANTS (M) AT DIFFERENT STAGES OF DEVELOPMENT GROWN IN SOLUTIONS WITH EITHER AMMONIUM OR NITRATE NUTRITION, TOGETHER WITH VALUES OF STANDARD DEVIATION ( $\sigma$ ), COEFFICIENT OF VARIABILITY (C), AND PROBABLE ERROR (E) OF THE MEAN

DATE	AMMONIUM N.				NITRATE N.			
	M	$\sigma$	C	E	M	$\sigma$	C	E
	<i>gm.</i>		%		<i>gm.</i>		%	
Dec. 26 .....	124	19.6	15.8	$\pm$ 3.8	134	10.4	8.0	$\pm$ 2.0
Jan. 7 .....	130	20.2	15.5	$\pm$ 3.8	141	14.7	10.5	$\pm$ 2.8
Jan. 21 .....	140	22.0	15.8	$\pm$ 4.1	153	16.0	10.4	$\pm$ 3.0
Feb. 4 .....	164	25.0	15.2	$\pm$ 4.7	175	17.1	9.8	$\pm$ 3.2
Feb. 18 .....	193	30.0	15.5	$\pm$ 5.7	203	22.0	10.7	$\pm$ 4.1
Feb. 25 .....	227	37.4	16.5	$\pm$ 7.3	237	33.0	13.9	$\pm$ 6.4
Mar. 11 .....	266	40.0	15.0	$\pm$ 7.8	276	34.1	12.3	$\pm$ 6.6
Mar. 28 .....	320	47.0	14.9	$\pm$ 9.2	325	38.7	11.9	$\pm$ 7.6
Apr. 11 .....	368	51.0	13.9	$\pm$ 10.0	381	48.0	12.6	$\pm$ 9.4
Apr. 30 .....	430	56.7	13.2	$\pm$ 11.0	446	52.4	11.8	$\pm$ 10.2

In table III the mean weight values of the ammonium and nitrate series of plants together with their statistical treatment are presented. The variability (C) in the plants of both series ranged, throughout their entire growth, from 8 to 16 per cent. The slightly greater variability in the ammonium- than in the nitrate-supplied plants was caused in certain cases by an accidental infection by pythiaceous organisms which produced a greater amount of root rot in the ammonium than in the nitrate series. Differences between the two series of plants in rate and amount of growth are insignificant, indicating that either ammonium or nitrate salts promoted growth at practically the same rate, provided other conditions were favorable.

The volume of water absorbed from the nutrient solution for every gram of fresh tissue produced (the *transpiration intensity* of the plant) was greater in the ammonium than in the nitrate series as shown in figure 9. The values obtained agree fairly well with those obtained by KRAUSS (5). The difference in transpired water between the two lots of plants is correlated with the greater tissue succulence of plants of the ammonium series. The moisture content of the various sections is reported in figure 10. The values for the ammonium series are in general higher than those for the nitrate-supplied plants, indicating a greater degree of succulence for plants of the ammonium series.



The distribution of chlorophyll in the various sections of leaves varies considerably as shown in figure 11. The amounts for corresponding tissues are greater in 11-A than in 11-B indicating that ammonium more than nitrate nutrition favored the formation of chlorophyll. The factors responsible for greater amounts of chlorophyll in the plants of the ammonium than in those of the nitrate series probably are the greater amounts of absorbed

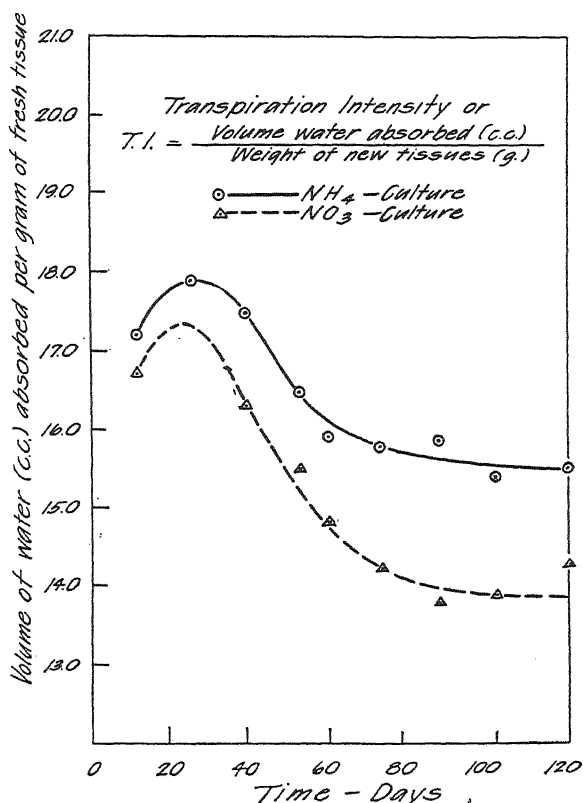


FIG. 9. Transpiration intensity of plants with ammonium and with nitrate nutrition.

nitrogen and the higher acidity in the nutrient solution and on the surface of the roots, caused at least in part by unequal absorption of ammonium and sulphate ions. Under such conditions iron and phosphorus as well as certain other elements would remain in solution and therefore be more available. The residual effect in the case of nitrate nutrition is that of decreased acidity owing to a more rapid absorption of the nitrate than the calcium ions.

With higher amounts of chlorophyll in the leaves synthesis of greater quantities of sugars might be anticipated. However, the quantities of sugars in the tissues of the ammonium series of plants with higher amounts of

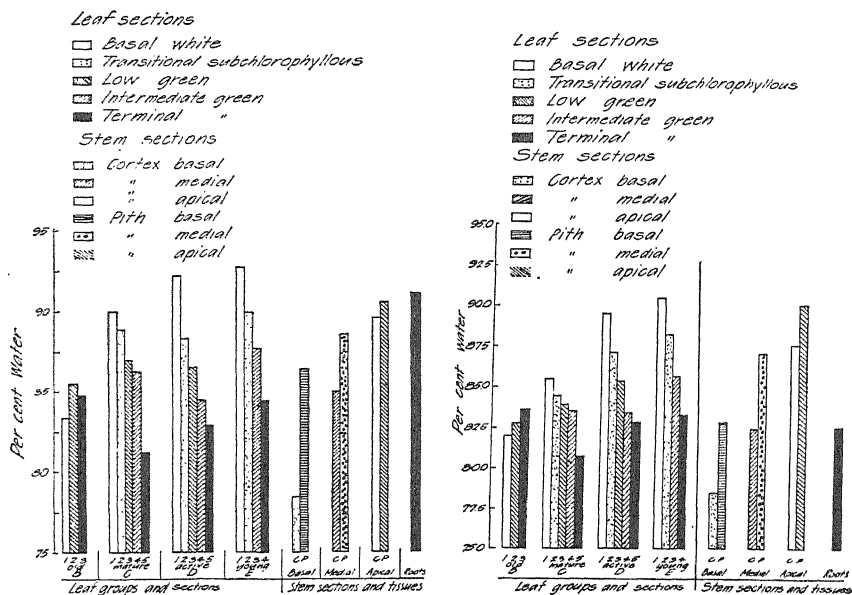


FIG. 10. Percentage water content of various sections of pineapple plants with ammonium nutrition (A), and with nitrate nutrition (B).

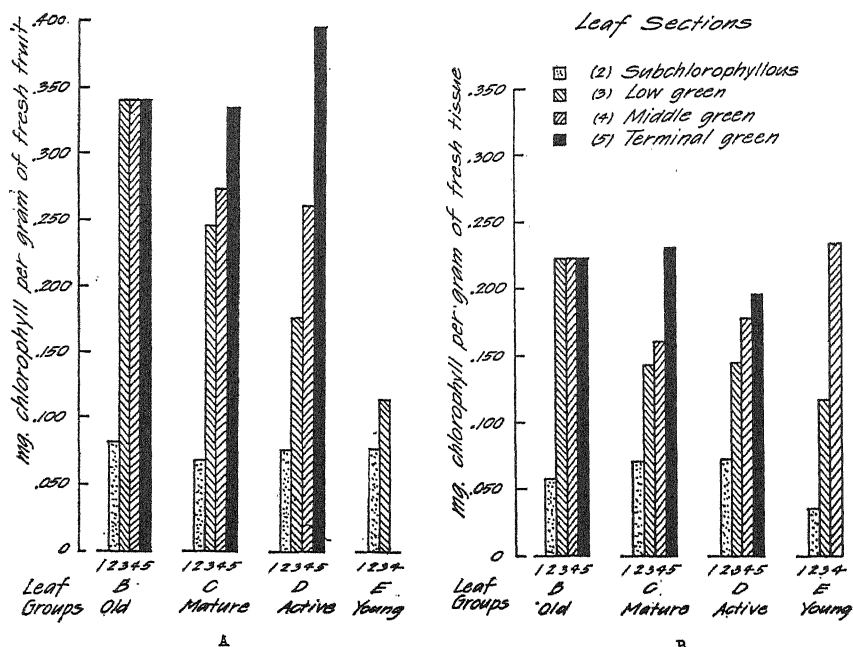


FIG. 11. Chlorophyll content of various leaf sections with ammonium nutrition (A), and nitrate nutrition (B).

chlorophyll were not any greater than those of the nitrate series. This may well have been caused in part by the great utilization of sugars, or their derivatives, in organic nitrogen synthesis, which, as will be subsequently shown, took place at a relatively higher rate in the leaves of the ammonium group.

#### RELATIVE ABSORPTION OF AMMONIUM AND NITRATE NITROGEN

Our discussion of the work of other investigators (13) has indicated that ammonium is a better source of nitrogen under field conditions than nitrate. In culture solutions STEWART, THOMAS, AND HORNER (14) have pointed out that nitrate in high, and ammonium in low concentrations were satisfactory sources. The analytical data of these investigators (p. 234, table 5), however, show that the quantities of nitrogen in the tissues of the plants grown either in high or low ammonium or nitrate cultures were consistently greater in the plants with ammonium nutrition, in spite of the adverse statements of these authors that ammonium in the "normal strength" culture was not readily utilized.

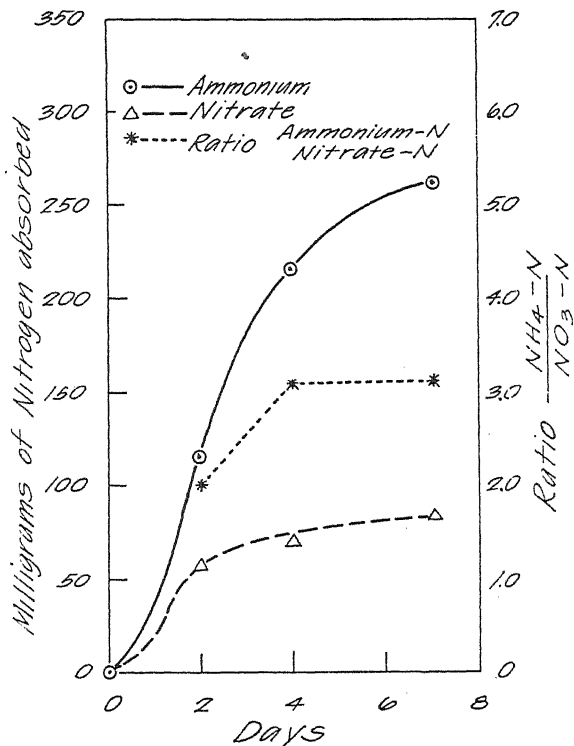


FIG. 12. Comparative absorption of ammonium and nitrate nitrogen by pineapple roots on a per plant basis.

Accordingly, an experiment on direct nitrogen absorption was established to measure the quantities of ammonium or nitrate nitrogen absorbed as such from the nutrient solution. Plants with highly uniform root and leaf systems were selected and placed in containers with either ammonium or nitrate nitrogen in equal amounts. Light and temperature conditions and the initial pH  $5.0 \pm 0.4$  were as nearly alike as possible in both cases. At intervals of 2, 4, and 7 days the quantities of nitrogen removed were determined by analysis of the residual culture solutions. The results obtained are reported in table IV, and figure 12.

TABLE IV

MEAN VALUES OF ABSORBED NITROGEN PER PLANT FROM SOLUTION CULTURES  
WITH EITHER AMMONIUM OR NITRATE NITROGEN

DAYS	ABSORBED NITROGEN AS		RATIO $\text{NH}_4\text{-N}$ $\text{NO}_3\text{-N}$
	AMMONIUM	NITRATE	
	<i>mg.</i>	<i>mg.</i>	
2 .....	116	57	2.0
4 .....	215	69	3.1
7 .....	260	83	3.1

The values of absorption of either ammonium or nitrate nitrogen, as stated in table IV, do not apply to the entire growth cycle of the plant but only to that part between the second and third months after planting. That this rate of absorption is maintained throughout the entire growth cycle is very doubtful. Analytical data, however, have shown in certain of the cases already mentioned and in those reported below that the plants absorbed nitrogen more rapidly from the ammonium than from the nitrate cultures.

#### DISTRIBUTION OF THE ABSORBED NITROGEN IN THE PLANT

The terms inorganic and unassimilated nitrogen are synonymous. When inorganic nitrogen either as ammonium or nitrate enters the roots, it may be converted immediately into organic nitrogen or it may be translocated through the water conducting tissues to the stem and then to the leaves. Organic nitrogen is segregated for convenience on the basis of whether it is soluble or insoluble in water. Soluble organic nitrogen comprises such fractions as amide (glutamine and asparagine), mono-amino (mostly nitrogen of alpha-amino acids), basic (mostly nitrogen from diamino acids) and rest nitrogen (undetermined compounds). Insoluble organic nitrogen is composed almost exclusively of protein. In the analyses which follow, proteins have been hydrolysed with sulphuric acid and the resulting fractions determined separately.

In tables V and VI are presented all the data on the analyses of the various tissues of both series of plants. Tables VII and VIII contain practically the same data as V and VI except that the values of the different nitrogenous fractions are reported on a percentage basis, which facilitates comparison. Figures 13-A to 18-A illustrate graphically the quantities of the different

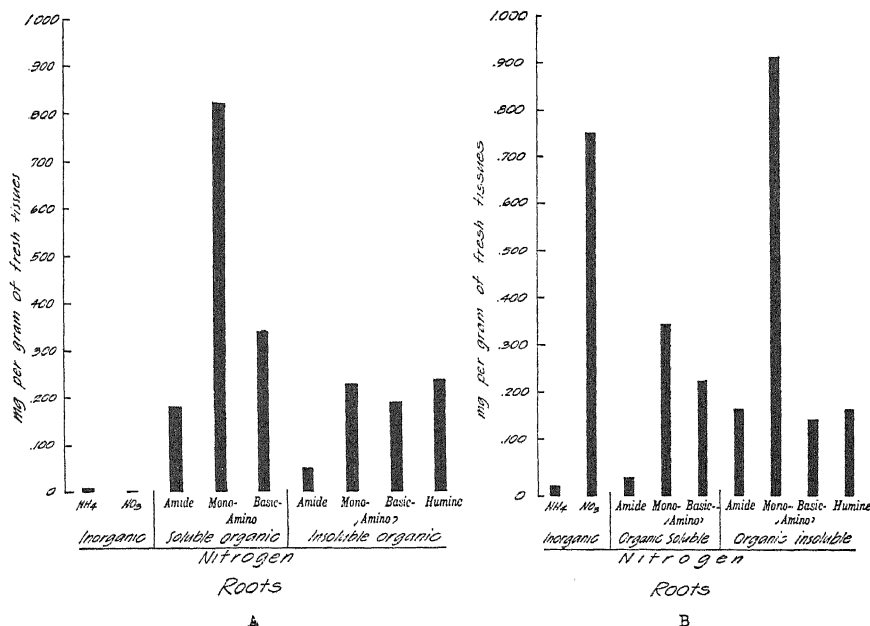


FIG. 13. Distribution of inorganic, soluble and insoluble organic nitrogen fractions in the roots of plants with ammonium nutrition (A), and nitrate nutrition (B).

fractions of nitrogen in the various sections of the plants supplied with ammonium nitrogen, and figures 13-B to 18-B illustrate the nitrogen composition of plants supplied with nitrate nitrogen.

**Roots.**—The outstanding results in the chemical composition of the root tissues of the ammonium and nitrate cultures (fig. 13) are: (a), very small amounts of ammonium in the roots of plants supplied with this nutrient, indicating a very high rate of assimilation of ammonium; (b), very great quantities of nitrate in the roots of plants with nitrate nutrition, indirectly indicating that it is absorbed fully but assimilated at a relatively slow rate; (c), large amounts of soluble organic nitrogen in the roots of the ammonium-supplied plants and comparatively small quantities in those of the nitrate cultures, results clearly in harmony with the relative amounts of (absorbed) ammonium and nitrates; and (d), very great quantities of insoluble organic nitrogen in the plants receiving nitrate nutrition and the low quantities in those of the ammonium cultures suggesting in the latter

TABLE V

DISTRIBUTION OF INORGANIC, SOLUBLE ORGANIC AND INSOLUBLE ORGANIC NITROGEN FRACTIONS IN THE DIFFERENT SECTIONS OF THE LEAVES, STEM AND ROOTS OF *Ananas comosus* (L.) MERR. GROWN IN AMMONIUM SOLUTION CULTURES

PLANT TISSUES	MILLIGRAM OF NITROGEN PER GRAM OF FRESH TISSUES										TOTAL ORGANIC
	INORGANIC			SOLUBLE ORGANIC			INSOLUBLE ORGANIC				
	AMMO- NIUM	NI- TRATE	GLU- TAMINE	ASPAR- AGINE	MONO- AMINO	BASIC	AMIDE	MONO- AMINO	BASIC	HUMINE	
Leaves: *	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
B-1 (base)	0.003	0	0.003	0.132	0.240	0.285	0.128	0.243	0.112	0.189	1.332
2	0.006	0	0.002	0.094	0.200	0.253	0.067	0.231	0.071	0.115	1.032
3 (tip) ..	0.008	0	0.007	0.144	0.470	0.534	0.074	0.461	0.141	0.211	2.041
C-1 (base)	0.002	0	0.038	0.008	0.286	0.202	0.114	0.290	0.040	0.054	1.032
2	0.002	0	0.027	.....	0.135	0.142	0.107	0.472	0.101	0.088	1.082
3	0.001	0	0.058	0.060	0.260	0.188	0.175	1.060	0.195	0.168	2.164
4	0.002	0	0.013	0.030	0.382	0.303	0.278	1.448	0.290	0.202	3.026
5 (tip) ..	0.002	0	0.037	0.064	0.378	0.303	0.320	1.630	0.317	0.222	3.231
D-1 (base)	0.002	0	0.030	0.112	0.490	0.371	0.099	0.343	0.074	0.054	1.573
2	0.001	0	0.026	0.008	0.164	0.148	0.129	0.626	0.108	0.088	1.297
3	0.002	0	0.028	0.020	0.233	0.236	0.266	1.185	0.208	0.135	2.311
4	0.002	0	0.016	0.060	0.370	0.384	0.288	1.360	0.283	0.189	2.950
5 (tip) ..	0.001	0	0.022	0.046	0.500	0.445	0.335	1.930	0.310	0.243	3.830
E-1 (base)	.....	0	0.011	0.236	0.566	0.512	0.093	0.410	0.115	0.086	2.029
2	0.001	0	0.018	0.204	0.328	0.258	0.070	0.301	0.099	0.099	1.377
3	0.002	0	0.014	0.186	0.572	0.466	0.147	0.505	0.168	0.199	2.255
4 (tip) ..	0.008	0	0.008	0.180	0.792	0.552	0.096	0.589	0.218	0.294	2.729
Stem: *											
B-Cortex	0.009	0	0.009	0.144	0.206	0.224	0.051	0.231	0.099	0.179	1.143
Pith	0.005	0	0.010	0.204	0.193	0.249	0.042	0.083	0.048	0.074	0.903
C-Cortex	0.008	0	0.011	0.236	0.480	0.441	0.042	0.269	0.102	0.173	1.744
Pith	0.013	0	0.007	0.180	0.497	0.459	0.045	0.115	0.048	0.077	1.428
D-Cortex	0.009	0	0.071	0.172	0.704	0.378	0.183	0.572	0.128	0.067	2.275
Pith	0.005	0	0.056	0.172	0.656	0.310	0.152	0.408	0.101	0.034	1.889
Roots	0.025	0	0.009	0.338	0.653	0.340	0.051	0.230	0.090	0.138	1.849

\* For a more detailed description of plant parts see text.

TABLE VI

DISTRIBUTION OF INORGANIC, SOLUBLE ORGANIC AND INSOLUBLE ORGANIC NITROGEN FRACTIONS IN THE DIFFERENT SECTIONS OF THE LEAVES, STEM AND ROOTS OF *Ananas comosus* (L.) MERR. GROWN IN NITRATE SOLUTION CULTURES

MILLIGRAM OF NITROGEN PER GRAM OF FRESH TISSUES												
PLANT TISSUES	INORGANIC			SOLUBLE ORGANIC			INSOLUBLE ORGANIC					TOTAL ORGANIC
	AMMO- NIUM	NI- TRATE	GLU- TAMINE	ASPAR- AGINE	MONO- AMINO	BASIC	AMIDE	MONO- AMINO	BASIC	HUMINE		
Leaves : *	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	
B-1 (base)	0.005	0.024	0.053	0.106	0.036	0.095	0.068	0.557	0.050	0.150	1.115	
2	0.011	0.006	0.053	0.114	0.013	0.074	0.059	0.700	0.040	0.072	1.125	
3 (tip) ..	0.007	0.000	0.049	0.106	0.023	0.092	0.099	0.714	0.167	0.120	1.370	
G-1 (base)	0.007	0.212	0.049	0.106	0.046	0.167	0.065	0.462	0.059	0.086	1.040	
2	0.006	0.056	0.058	0.116	0.031	0.116	0.068	0.492	0.044	0.086	1.011	
3	0.007	0.004	0.058	0.094	0.031	0.112	0.086	0.700	0.194	0.136	1.411	
4	0.005	0.000	0.058	0.100	0.011	0.115	0.119	0.980	0.307	0.153	1.843	
5 (tip) ..	0.006	0.000	0.058	0.132	0.012	0.157	0.136	1.060	0.375	0.200	2.122	
D-1 (base)	0.022	0.179	0.058	0.226	0.026	0.133	0.063	0.307	0.043	0.067	0.923	
2	0.007	0.144	0.053	0.120	0.026	0.121	0.100	0.590	0.067	0.086	1.163	
3	0.007	0.008	0.056	0.136	0.031	0.157	0.125	0.920	0.231	0.125	1.781	
4	0.008	0.000	0.053	0.130	0.009	0.148	0.183	1.160	0.260	0.176	2.121	
5 (tip) ..	0.008	0.000	0.008	0.042	0.103	0.157	0.275	1.264	0.345	0.187	2.381	
E-1 (base)	0.006	0.063	0.014	0.092	0.250	0.243	.....	.....	.....	.....	.....	
2	0.009	0.047	0.011	0.056	0.149	0.138	.....	.....	.....	.....	.....	
3	0.009	0.006	0.006	0.040	0.104	0.108	.....	.....	.....	.....	.....	
4 (tip) ..	0.010	0.000	0.006	0.052	0.101	0.133	0.167	1.220	0.380	0.170	2.229	
Stem : *												
B-Cortex ..	0.007	0.242	0.017	0.040	0.184	0.152	0.084	0.435	0.113	0.106	1.131	
Pith .....	0.007	0.234	0.009	0.022	0.285	0.194	0.075	0.352	0.056	0.052	1.045	
C-Cortex ..	0.018	0.280†	0.017	0.084	0.466	0.296	0.097	0.512	0.115	0.075	1.662	
Pith .....	0.016	0.375	0.018	0.106	0.613	0.436	0.087	0.474	0.100	0.061	1.895	
D-Cortex ..	0.008	0.312	0.028	0.134	0.439	0.355	0.089	0.486	0.134	0.046	1.711	
Pith .....	0.037	0.469	0.027	0.258	0.564	0.426	0.125	0.640	0.167	0.064	2.271	
Roots .....	0.022	0.750	0.013	0.052	0.319	0.227	0.167	0.916	0.141	0.163	1.998	

\* For a more detailed description of plant parts see text.

† Obtained with the xylenol method.

TABLE VII

GRAMS OF INORGANIC, SOLUBLE ORGANIC, INSOLUBLE ORGANIC AND TOTAL NITROGEN PER GRAM OF FRESH WEIGHT IN THE DIFFERENT SECTIONS OF THE PLANT, TOGETHER WITH PERCENTAGE VALUES OF THE SAME AND OF THEIR FRACTIONS AS FOUND IN PINEAPPLE PLANTS GROWN IN SOLUTION CULTURES CONTAINING AMMONIUM NITROGEN

PLANT ISSUES	INORGANIC NITROGEN				SOLUBLE ORGANIC NITROGEN				INSOLUBLE ORGANIC NITROGEN							
	% OF INORGANIC		TOTAL	% OF TOTAL	% OF SOLUBLE ORGANIC				TOTAL	% OF TOTAL	% OF INSOLUBLE					
	AMMO- NIUM	NITRATE			GLUTA- MINE	ASPAR- AGINE	MONO- AMINO	BASIC			AMIDE	MONO- AMINO	BASIC	HUMINE		
	%	%	mg.	%	%	%	%	mg.	%	%	%	%	%	%	%	%
1 (base)	100	0	0.003	0.2	0.4	10.0	46.4	0.660	49.5	19.05	36.15	28.15	50.3			
2	100	0	0.006	0.6	0.3	8.5	45.0	0.948	52.8	13.80	47.80	23.75	46.6			
3 (tip)	100	0	0.008	0.3	0.5	6.3	47.0	1.154	56.4	8.30	52.00	23.80	43.3			
1 (base)	100	0	0.002	0.2	7.1	0.8	54.3	0.534	51.6	22.90	58.30	10.80	48.2			
2	100	0	0.002	0.2	8.6	3.2	43.0	0.314	28.8	13.95	61.45	11.45	71.0			
3	100	0	0.001	0.1	8.9	4.6	51.2	0.366	26.1	11.00	66.30	10.50	1.598			
4	100	0	0.002	0.1	2.1	2.4	46.7	0.708	23.4	12.50	65.40	9.10	73.8			
5 (tip)	100	0	0.002	0.1	5.0	4.3	49.9	0.742	22.9	12.90	65.50	8.90	76.5			
1 (base)	100	0	0.002	0.1	3.0	5.5	54.5	1.003	63.7	17.40	60.16	9.47	77.0			
2	100	0	0.001	0.1	7.5	1.1	48.6	0.346	26.6	13.50	65.90	9.20	36.2			
3	100	0	0.002	0.2	5.4	1.9	47.0	0.604	25.4	14.80	66.10	7.50	73.3			
4	100	0	0.002	0.1	1.9	3.6	48.2	0.830	26.9	13.55	64.20	8.90	74.6			
5 (tip)	100	0	0.001	0.0	2.2	2.2	51.6	1.012	26.4	11.90	68.50	8.60	73.1			
(base)	100	0	0.001	0.1	0.9	8.9	51.6	1.325	65.3	13.20	58.30	12.20	73.6			
2	100	0	0.001	0.1	2.2	12.6	53.2	0.808	58.6	12.30	53.00	17.35	34.7			
3	100	0	0.002	0.1	1.1	7.5	53.8	1.238	54.8	14.40	49.75	16.30	41.3			
4 (tip)	100	0	0.008	0.3	0.6	5.9	57.5	1.532	56.0	8.10	49.20	24.50	45.1			
(base)	100	0	0.009	1.2	1.4	12.4	47.7	0.583	50.3	9.10	41.20	32.00	48.7			
2	100	0	0.005	0.5	1.4	15.6	45.0	0.656	72.3	17.00	33.60	30.00	27.2			
3 (tip)	100	0	0.008	0.5	0.9	10.1	51.2	1.168	66.3	7.17	45.90	29.50	33.2			
4	100	0	0.013	0.9	0.5	7.9	51.4	1.143	79.3	15.60	40.40	17.80	19.8			
5 (tip)	100	0	0.009	0.4	5.4	6.5	59.6	1.325	58.0	18.30	60.20	7.05	41.6			
6 (tip)	100	0	0.005	0.3	4.6	7.2	62.2	1.194	63.0	21.90	58.70	4.90	36.7			
7 (tip)	100	0	0.025	1.4	0.6	12.6	61.4	1.340	71.5	10.00	45.20	27.10	50.9			

For a more detailed description of plant parts see text.



TABLE VIII

AMS OF INORGANIC, SOLUBLE ORGANIC, INSOLUBLE ORGANIC, AND TOTAL NITROGEN PER GRAM OF FRESH WEIGHT IN THE DIFFERENT SECTIONS OF THE PLANT, TOGETHER  
PERCENTAGE VALUES OF THE SAME AND OF THEIR FRACTIONS AS FOUND IN PINEAPPLE PLANTS GROWN IN SOLUTION CULTURES CONTAINING NITRATE NITROGEN

ANT SUES	INORGANIC NITROGEN				SOLUBLE ORGANIC NITROGEN						INSOLUBLE ORGANIC NITROGEN					
	% OF INORGANIC		TOTAL	% OF TOTAL	% OF SOLUBLE ORGANIC				TOTAL	% OF TOTAL	% OF INSOLUBLE				TOTAL	% OF TOTAL
	AMMO- NIUM	NITRATE			GLUTA- MINE	ASPAR- AGINE	MONO- AMINO	BASIC			AMIDE	MONO- AMINO	BASIC	HUMINE		
* (base)	% 17.0 65.0 100.0	% 83.0 35.0 0.0	mg. 0.029 0.017 0.007	% 2.7 1.5 0.6	% 18.30 20.90 18.15	% 18.30 22.40 19.60	% 30.70 27.60 28.10	% 32.70 29.10 34.00	% 25.30 22.20 19.60	mg. 0.290 0.254 0.270	% 8.25 6.78 9.00	% 67.50 80.40 65.00	% 6.06 4.59 15.10	% 18.17 8.27 10.90	mg. 0.825 0.871 1.100	% 72.0 76.3 79.8
(base)	3.0 10.0 64.0 100.0 100.0	97.0 90.0 36.0 0.0 0.0	0.219 0.062 0.011 0.005 0.006	10.6 5.8 0.8 0.1 0.1	13.30 18.07 19.66 20.40 16.50	14.40 18.08 15.94 17.60 17.66	26.90 27.70 26.45 21.50 20.90	45.40 36.15 37.95 40.50 44.80	29.20 29.90 20.70 15.40 16.50	0.368 0.321 0.295 0.284 0.351	9.67 9.85 7.72 7.63 7.68	68.80 71.30 62.80 62.90 60.00	8.78 6.38 17.40 19.70 21.18	12.80 12.46 12.20 9.85 11.28	0.672 0.690 1.116 1.559 1.771	60.2 64.3 78.5 84.5 83.4
(base)	19.0 5.0 47.0 100.0 100.0	89.0 95.0 53.0 0.0 0.0	0.201 0.101 0.015 0.008 0.008	17.9 11.4 0.8 0.3 0.3	13.10 16.55 14.73 15.50 2.63	25.50 18.75 17.90 19.00 6.77	31.40 26.90 26.00 22.20 40.00	30.00 37.80 41.30 43.30 50.60	39.40 24.40 21.20 16.10 13.00	0.443 0.320 0.380 0.342 0.310	13.12 11.86 8.92 10.28 13.27	64.00 70.00 65.70 65.20 61.10	8.96 7.95 16.50 14.60 16.65	13.95 10.20 8.92 9.90 9.04	0.480 0.843 1.401 1.779 2.071	42.7 64.2 78.0 83.6 86.7
(base)	9.0 16.0 60.0 100.0	91.0 84.0 40.0 0.0	0.069 0.056 0.015 0.010	..... ..... ..... .....	2.30 3.10 2.30 2.00	7.70 7.90 7.70 8.90	49.40 50.00 48.20 43.50	40.60 39.00 41.80 45.60	..... ..... ..... 13.04	0.599 0.354 0.258 0.292	..... ..... ..... 8.62	..... ..... ..... 63.00	..... ..... ..... 19.60	..... ..... ..... 8.78	..... ..... ..... 1.937	..... ..... ..... 86.6
(tex h	3.0 3.0	97.0 97.0	0.249 0.241	18.1 18.7	4.31 1.75	5.09 2.15	52.00 58.00	38.60 38.10	28.50 39.70	0.393 0.510	11.35 14.00	59.00 65.80	15.30 10.48	14.35 9.72	0.738 0.535	53.4 41.6
(tex h	6.0 4.0	94.0 96.0	0.298 0.391	15.3 17.1	1.97 1.58	4.87 4.52	58.90 56.80	34.30 37.10	44.00 51.30	0.868 1.173	12.20 12.04	64.00 65.40	14.40 13.82	9.40 8.44	0.799 0.722	40.7 31.6
(tex h	2.4 7.0	97.6 93.0	0.320 0.506	15.8 18.1	2.93 2.10	7.00 10.10	53.00 54.25	37.15 33.50	47.00 46.00	0.956 1.275	11.75 12.70	64.40 64.30	17.75 16.60	6.10 6.40	0.755 0.996	37.2 35.9
	2.8	97.2	0.772	28.0	2.10	4.25	56.50	37.15	22.00	0.611	12.00	66.00	10.15	11.75	1.387	50.0

For a more detailed description of plant parts see text.

case the possible absence of certain substances essential for protein synthesis. It is difficult to explain the presence of comparatively great quantities of protein nitrogen in the presence of very small amounts of soluble organic nitrogen and of a large reserve of nitrate nitrogen. The authors' opinion is that such proteins are synthesized from soluble organic nitrogen translocated from the leaves through the stem and are probably not newly synthesized by the root tissues. Further studies are necessary to prove or disprove this contention. It is also possible that the great amounts of protein in the plants of the nitrate cultures are caused by a greater condensation of amino acids to protein under conditions of high sugar content. Total nitrogen values reported in tables VII and VIII are about 7.5 per cent. greater for the nitrate than for the ammonium-supplied plants, possibly on account of the accumulation of greater quantities of proteins.

**STEMS.**—The quantities of ammonium in the stems of the ammonium-supplied plants, as shown in figure 14-A, are exceedingly small and no nitrate is present. Explanations for both conditions have been offered in the section on roots and they apply equally well in the present case.

The values for soluble organic nitrogen are relatively high, indicating a high rate of translocation from the roots and a transient accumulation in the stem. It will be noted that the amounts of the different fractions are greater in the apical (D + E) than in the intermediate (C) and the basal (A + B) sections of the stem. It is possible that part of the soluble organic nitrogen fraction has been translocated to the stem from the leaves.

The relative amounts of protein (insoluble nitrogen) in the stem are comparatively moderate, but greater than in the roots and smaller than in the C and D groups of leaves. Carbohydrates or their derivatives are essential for protein synthesis from inorganic nitrogen and they are found in greater amounts in the stem than in the roots.

The amount of nitrate nitrogen in the stems of the plants of the nitrate-supplied series is comparatively high, according to the data shown in figure 14-B, which indicates a direct movement of this substance from the roots. It also indicates that its rate of assimilation in the stem is very low. Ammonium nitrogen is found only in traces in the various tissues, the possible sources of which have been already mentioned.

The amounts of soluble organic nitrogen in the stem are very great as compared to those in the roots and leaves. We cannot explain this condition in any other way except that the soluble fractions of organic nitrogen have been translocated from the leaves where nitrate nitrogen is assimilated very readily. Protein nitrogen in the stem is low when compared with that in the roots and leaves. The presence of great amounts of soluble organic and of small amounts of insoluble organic nitrogen indicate that the later steps in protein synthesis do not take place rapidly in the stem. Although

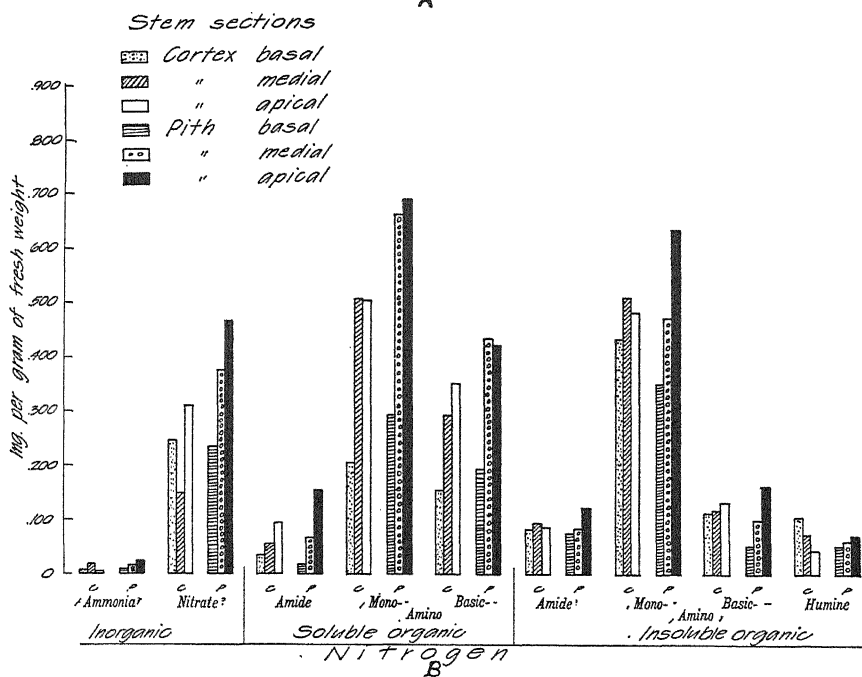
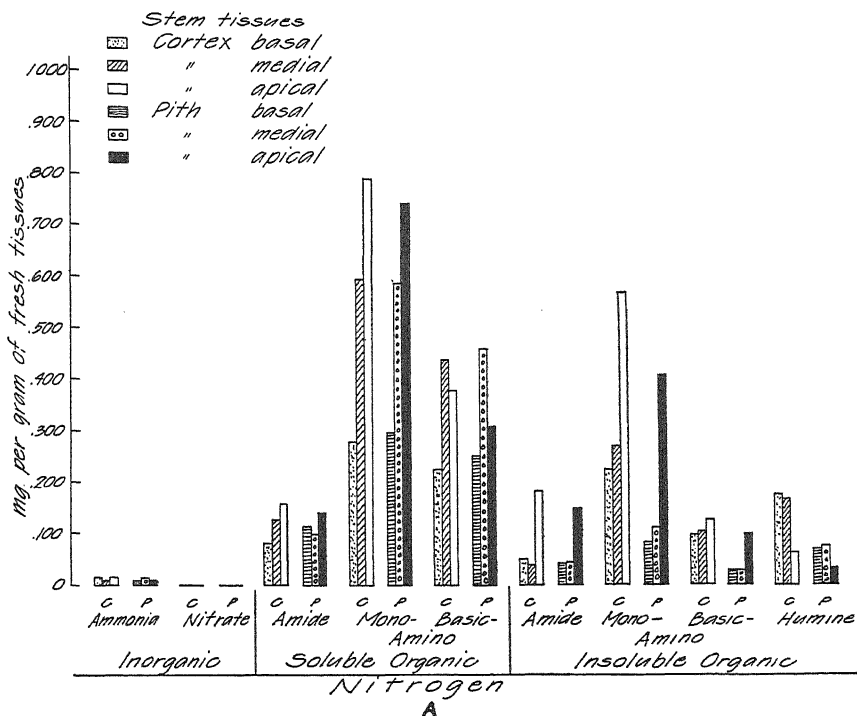


FIG. 14. Distribution of inorganic, soluble and insoluble organic nitrogen fractions in the various sections of the cortex and pith of the stem of plants with ammonium nutrition (A), and nitrate nutrition (B).

no satisfactory explanation can be offered, it may be pointed out that nitrate-supplied plants of many species display similar differences in the protein content of leaves, stems, and roots.

In a comparative study of the distribution of the soluble and insoluble fractions of organic nitrogen in the stem tissues, very little difference can be found between the plants of the ammonium and those of the nitrate series, possibly because of the position of the stem between roots and leaves, the two most active assimilating organs and sources of organic nitrogen. Total organic nitrogen as shown in tables VII and VIII and in figure 14 was consistently greater in the ammonium than in the nitrate-supplied plants.

#### LEAVES.—

*Old leaves (group B).*—This group of leaves represents planting-material-leaves which are composed of relatively old and partly senile tissues. The composition of the tissues of these leaves, therefore, cannot be compared with that of the other leaf-groups. The data in figure 15-A, show

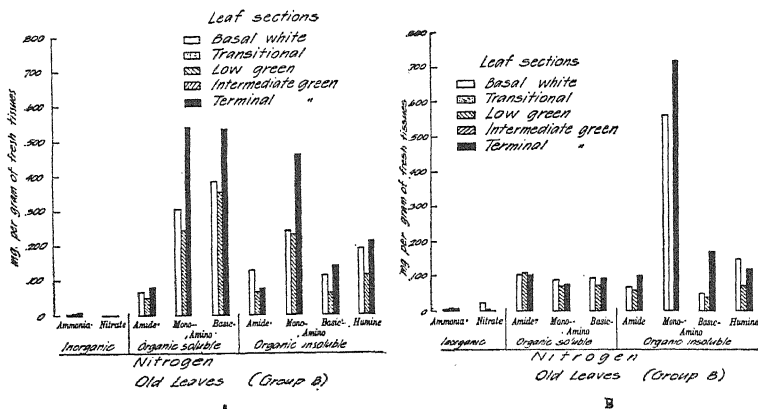


FIG. 15. Distribution of inorganic, soluble and insoluble organic nitrogen fractions in the various sections of old leaves (group B) of plants with ammonium nutrition (A), and nitrate nutrition (B).

the distribution of the different nitrogen fractions in the old leaves (group B) of the ammonium-supplied plants. There is indicated a very small concentration of ammonium and no nitrate in the various leaf sections. The amounts of soluble organic nitrogen are comparatively high, while those of protein or insoluble nitrogen are low. It is possible that the high values of soluble organic nitrogen represent translocated nitrogen which, owing to cessation of growth of the old leaves, is not utilized in the formation of new tissues; or it may represent decomposition products which are released from the proteins of senile tissues by hydrolysis.

The data presented in figure 15-B for the nitrate-supplied plants show

very small amounts of ammonium nitrogen in the white basal leaf tissue, but somewhat greater quantities of nitrate. Nitrate nitrogen has apparently entered the basal non-chlorophyllous tissues after passing through the roots and stem. However, it disappeared, presumably, as it penetrated into the chlorophyllous tissues.

Soluble organic nitrogen occurred in relatively small amounts in the old leaves of the nitrate-supplied plants. Amide nitrogen constituted a much greater portion of the soluble organic nitrogen fraction than mono-amino nitrogen. The latter was associated with plants of the ammonium rather than with those of the nitrate series, in the leaves concerned.

The concentration of protein nitrogen was, as in all the cases of nitrate nutrition so far studied, comparatively great. No statement can explain satisfactorily this condition in the absence of greater and more detailed data. Total organic nitrogen values were, on an average, 25 per cent. higher in the plants grown in the cultures with ammonium than in those with nitrate as figure 19 and tables VII and VIII show.

*Mature leaves (group C).*—This group represents the first leaves produced by pineapple plants after planting which in order of development are the oldest excepting those initially present on the planting-material piece. On plants five months old the number of such leaves is about 6, while in older plants it increases. The analytical data presented in figure 16-A show

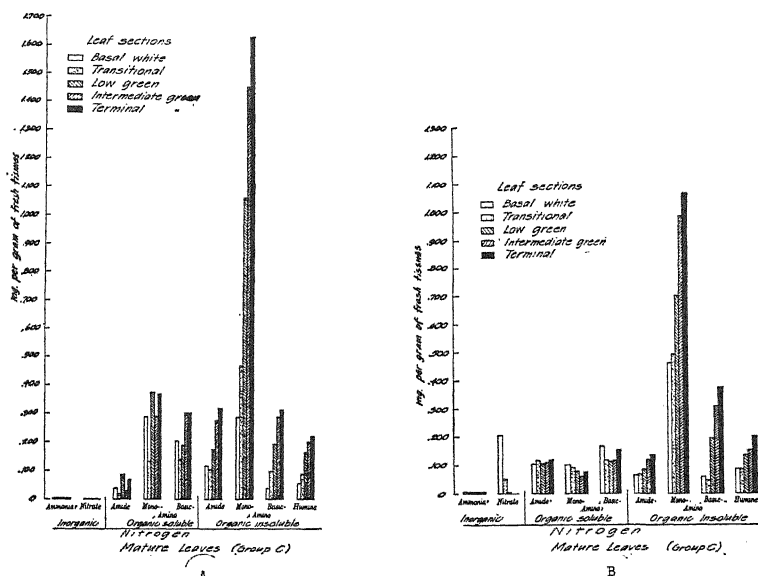


FIG. 16. Distribution of inorganic, soluble and insoluble organic nitrogen fractions in the various sections of mature leaves (group C) plants with ammonium nutrition (A), and nitrate nutrition (B).

that for ammonium-supplied plants, the amounts of ammonium nitrogen in the various sections of the old leaves were exceedingly small, while nitrate was entirely absent. Soluble organic nitrogen occurred in comparatively moderate concentrations with the mono-amino fraction present in greatest amounts. The insoluble organic nitrogen content was very high. The conditions favoring the synthesis of this fraction were probably very good in the green tissues of the old leaves where sugars, essential for protein synthesis, were present in sufficient quantities.

The quantities of ammonium nitrogen, as shown in figure 16-B, were very small for nitrate-supplied plants, whereas those of nitrate were exceedingly high in the basal, non-chlorophyllous tissues. The results obtained for inorganic nitrogen are similar to those for the older leaf group B except that the concentration of nitrate in the leaves of group C was considerably greater than in group B, owing probably to proportionally larger amounts of non-chlorophyllous tissues in the leaf bases of the group C plants and to more direct conduction, the latter point to be discussed later. The comparatively low plane of nitrate assimilation in the white basal tissue, a condition favoring the accumulation of nitrate in this region of the leaf, has been discussed previously. Soluble organic nitrogen occurred in relatively small amounts, with a trend of distribution of its different fractions similar to that in leaf group B.

Insoluble organic nitrogen in the old C leaves was very high and considerably higher than in the somewhat older B group of leaves. The conditions which favor the formation of great amounts of this form of nitrogen are an abundance of carbohydrates and certain other substances.

Comparative studies show that the amounts of insoluble organic nitrogen were greater in the leaves of the plants with ammonium than in those with nitrate nutrition as figure 16 shows. This condition was associated with a higher rate of absorption and assimilation of ammonium than of nitrate and also with the greater amounts of chlorophyll in the leaves of the plants with ammonium nutrition. Total organic nitrogen values, as reported in tables VII and VIII and figure 19 were, except for the basal white tissue, considerably higher in the plants supplied with ammonium than in those supplied with nitrate.

*Active leaves (group D).*—This group is composed of the longest leaves on the plant which are located between the fully grown and the more immature leaves. In four-months-old plants they number from 6 to 9. The analytical results for the various sections of the leaves of the ammonium-supplied plants are presented in figure 17-A. The quantities of ammonium were exceedingly small and nitrate was entirely lacking.

Soluble organic nitrogen occurred in relatively high concentration. The fractions, mono-amino and basic nitrogen, were considerably greater

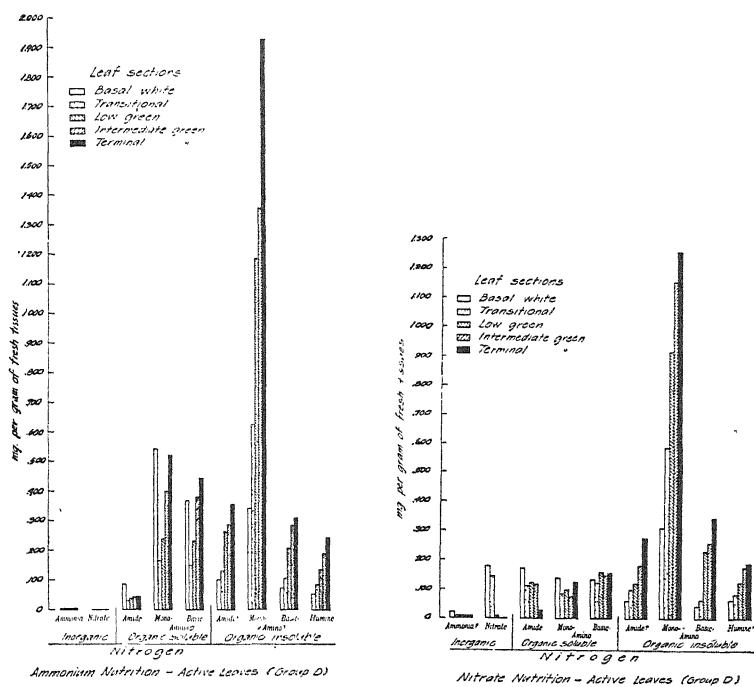


FIG. 17. Distribution of inorganic, soluble and insoluble organic nitrogen fractions in the various tissues of full grown active leaves (group D) of plants with ammonium nutrition (A), and nitrate nutrition (B).

than those of amide nitrogen. Insoluble organic nitrogen reached its greatest value in these leaves. Except for the accumulation of great amounts of protein the results are quite comparable with those of the somewhat older leaves of group C of the same series.

In the nitrate-supplied plants nitrate nitrogen occurred in comparatively great concentrations in the basal, non-chlorophyllous and in the transitional, sub-chlorophyllous tissues of the nearly fully expanded active leaves of the D group according to the data shown in figure 17-B. In the chlorophyllous tissues nitrate disappeared very readily, presumably owing to rapid synthesis to organic nitrogen. Ammonium nitrogen was present in small concentrations; its sources of derivation have been mentioned. The quantities of soluble organic nitrogen were moderate, the amide-nitrogen fraction being of approximately the same magnitude as the mono-amino and basic fractions. Protein content was very high, reaching its maximum concentration in the nitrate series in the D group of leaves.

Contrasting the protein content in the various sections of the large active leaves of group D of both series in figure 17, we find that in the ammonium

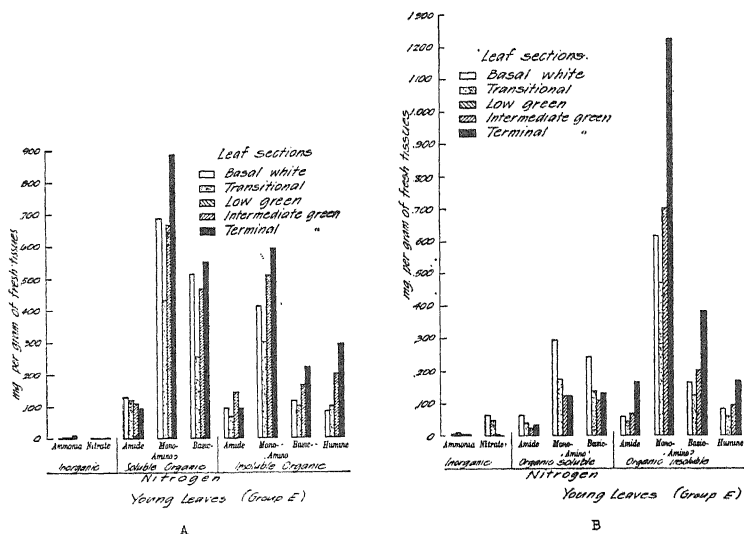


FIG. 18. Distribution of inorganic, soluble and insoluble organic nitrogen fractions in the various tissues of young leaves (group E) of plants with ammonium nutrition (A), and nitrate nutrition (B).

series the values are 30 per cent. higher than in the nitrate series. In this leaf group, as in the previous one, the causes for greater concentration of protein are a higher rate of ammonium than nitrate absorption, and concentrations of chlorophyll which possibly favored the synthesis of greater amounts of sugars in the plants of the ammonium than of the nitrate series. Total organic nitrogen values appearing in figure 19 and in tables VII and VIII were, on an average, about 27 per cent. higher in the ammonium than in the nitrate-supplied plants.

*Young leaves (group E).*—The leaves of this group are only partly developed, some having attained 80 and others 10 per cent. of their final growth. Associated with this condition the analytical data for ammonium-supplied plants, reported in figure 18-A show that the chemical composition of these leaves is, to a great extent, more like that of the immature (basal-non-chlorophyllous) tissues of leaf groups D and C than of the mature chlorophyllous tissues. Ammonium nitrogen was found in mere traces, except in the terminal tissues, where slightly greater amounts were observed, owing possibly to hydrolytic processes. Nitrate nitrogen, as in all cases of ammonium nutrition, was entirely lacking.

Soluble organic nitrogen and particularly the mono-amino and basic fractions were higher in these leaves than in any of the tissues so far studied.

Associated with proximity to chlorophyllous tissues, insoluble organic nitrogen was comparatively low, although higher than in the roots.



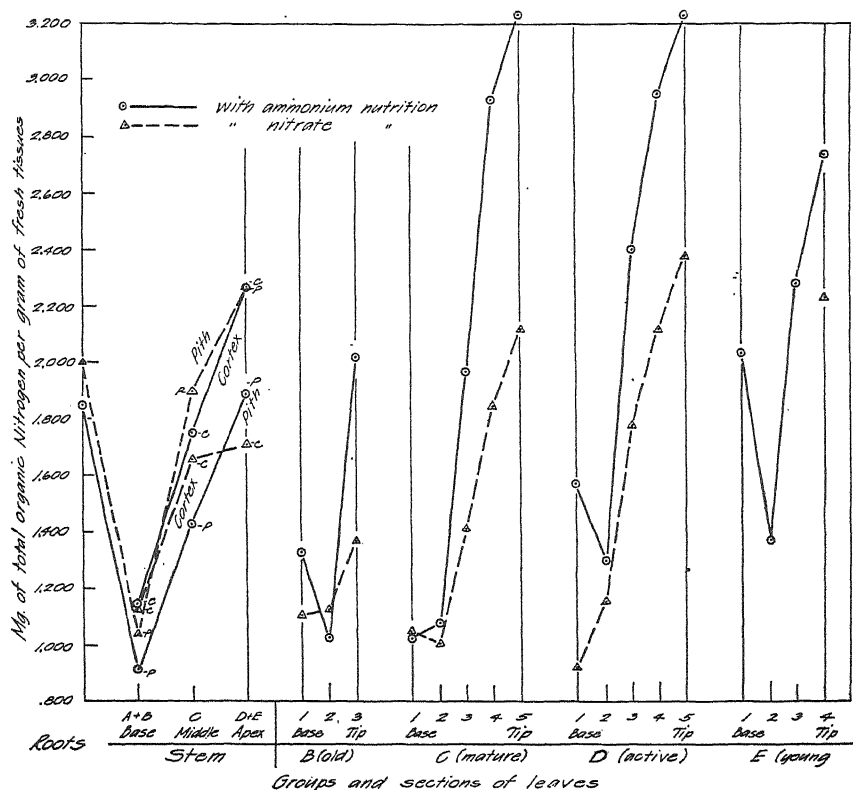


FIG. 19. Comparative distribution of total organic nitrogen in the various tissues of plants with either ammonium or nitrate nutrition.

According to the data shown in figure 18-B for nitrate-supplied plants, ammonium nitrogen was found in very small, while nitrate nitrogen was found in moderate, quantities. Of the soluble organic nitrogen fractions, glutamine was found in very small amounts while asparagine was found in appreciably greater ones. Mono-amino nitrogen values were significantly higher than in any other leaf groups of the same series, while those of basic nitrogen were approximately the same. The values for the insoluble nitrogen fractions are not complete on account of certain accidental losses of the samples during analysis.

Leaf section E-4 in the same figure shows greater amounts of insoluble nitrogen than the corresponding section of the ammonium series in figure 18-A.

#### DISTRIBUTION OF SUGARS

We are unable to report data on any other kind of carbohydrates than sugars, because the material to be used for analysis of starch, etc., was acci-

dentally subjected to comparatively high temperatures during dehydration and was found unfit for study. Sugars were determined in all cases on fresh tissue after thorough maceration in a brass mortar with quartz sand and subsequent extraction with ten volumes of water.

The distribution of reducing sugars and sucrose in the various sections of the leaves of the different groups as shown in table IX and figure 20

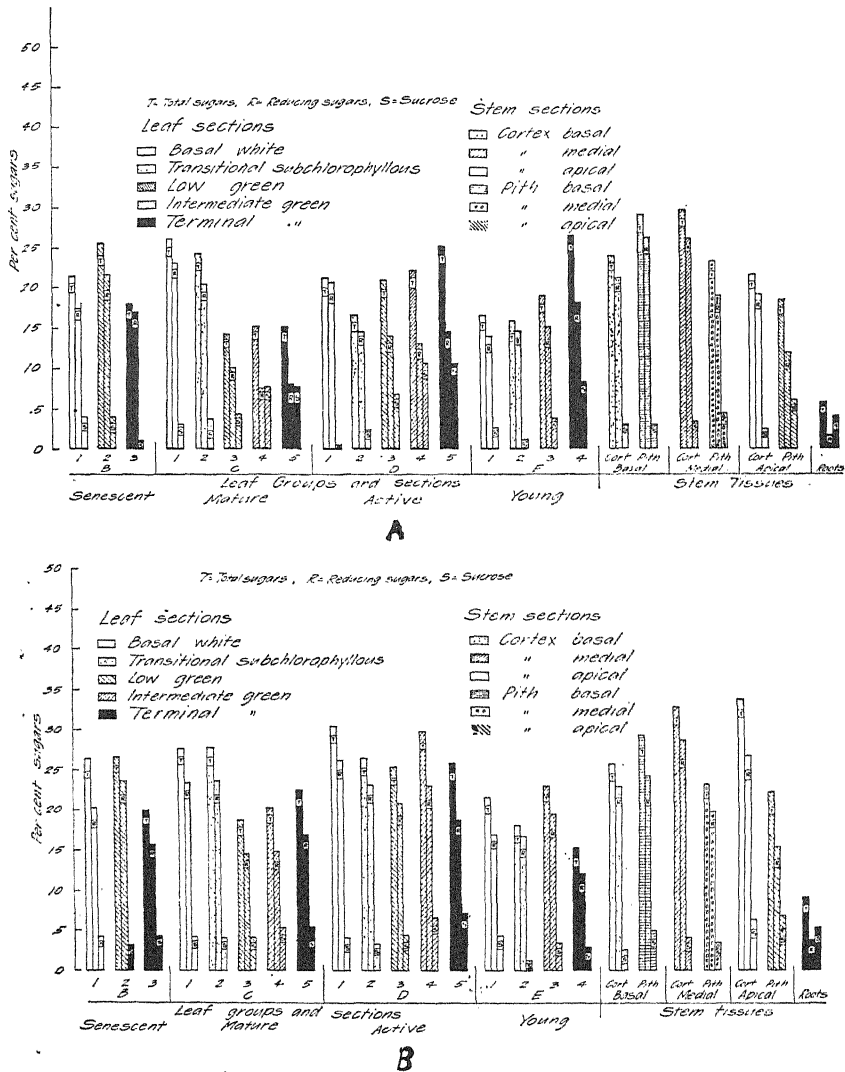


FIG. 20. Distribution of reducing sugars, sucrose, and total sugars in the various sections of the leaves of plants with ammonium nutrition (A), and nitrate nutrition (B).

differs considerably, but, with few exceptions, the order of distribution is practically the same for plants of the ammonium and nitrate series. Both kinds of sugars in the tissues of the old leaves of group B are irregularly distributed. This condition, owing to the presence of senile tissues, cannot be given serious consideration. In the sections of the mature group C leaves, reducing sugars and sucrose had definite order of distribution. The concentration of sucrose increased from the basal to the terminal tissue while reducing sugars decreased. In the nearly fully expanded active leaves of group D, the same order of distribution of both sugars was found as in the older C leaves. The concentration of reducing sugars in the terminal tissues of groups D and C were slightly higher than in the adjacent lower sections. The distribution of both reducing sugars and sucrose in the young leaves (group E) was somewhat different than in the two preceding groups of older leaves. The percentage of reducing sugars increased consistently from the basal to the terminal tissues. Sucrose, except in the basal tissues, followed the same order of distribution.

The order of distribution of sugars as observed in the chlorophyllous sections of the leaves of groups C and D was the same as that found in plants ranging in age from two to six months as observed in certain other studies (to be published later) where the distribution of sugars at different stages of plant growth was determined. In older plants the amounts of both reducing sugars and sucrose were higher in the chlorophyllous than in the sub-chlorophyllous and non-chlorophyllous tissues. Sucrose in plants of nearly all ages followed the same order of distribution, as has been found in the studies above mentioned.

In the tissues of the stem the order of distribution of reducing sugars decreased from base to apex in the pith, while in the cortex there was a tendency for the values to increase from the base until they reached the apex, where supposedly, they decreased on account of rapid utilization in new tissue formation. The distribution of total sugars in the tissues of the cortex of the stem is affected by translocation and the photosynthetic activity of the groups of leaves which serve directly the cells concerned. The concentration of sugars in the roots was comparatively very small. Sucrose was more abundant than reducing sugars. Not having direct evidence of the distribution of sucrose and reducing sugars in the stele and cortex of pineapple roots we cannot explain satisfactorily the difference in the quantities of these two sugars.

In somewhat related studies (13) conducted on the roots of *Pandanus veitchii* we found that the tissues of the cortex contained only reducing sugars and also that sucrose occurred exclusively in the tissues of the stele. As comparative data on the cortex and stele of pineapple roots are lacking we are unable to state definitely whether sucrose is exclusively restricted to

the tissues of the stele or whether it is distributed uniformly throughout the entire root. The presence of reserve or storage carbohydrate products, as sucrose, may explain at least in part, the rapid assimilation of ammonium and its conversion into soluble organic nitrogenous compounds.

The concentration of both reducing sugars and sucrose are from 10 to 25 per cent. greater in the plants of the nitrate than in those of the ammonium series, probably because of the relatively low rate of nitrogen assimilation. Both reducing sugars and sucrose are considerably lower in the roots of the plants of the ammonium than in those of the nitrate series. A condition probably associated with a greater rate of ammonium than of nitrate assimilation. The greater quantities of soluble organic nitrogen compounds in the roots of the plants with ammonium nutrition may account for the appreciably reduced values of sugars in the root tissues of this group of plants.

The difference in the distribution of sugars in the plants with ammonium and nitrate nutrition most worthy of consideration is that between the young tissues of the leaves, namely, the basal, non-chlorophyllous sections of leaf groups D and E and those of the apical section of the stem of the two respective series. These two groups of leaves, *viz.*, D and E, and the stem apex, not having attained complete development, are more actively engaged in growth processes and therefore more susceptible to biochemical variations than any of the other leaf groups or stem sections of the plant. The data in figure 20 show that the amounts of sugars in the basal non-chlorophyllous sections (no. 1) of the leaves and in the stem apex of the plants of the nitrate series are considerably greater than those of the corresponding sections of the ammonium series. These findings indicate that the rate of utilization of sugars in the sections of the leaves and stem under consideration was greater in the plants with ammonium than in those with nitrate nutrition. The distribution of sugars in the tissues of the terminal sections (nos. 4 and 5) of the E group of leaves is reversed. That is, the quantities of sugars in the terminal tissues of the leaves are greater in the plants with ammonium nutrition than in those with nitrate, indicating a greater rate of sugar utilization by the terminal leaf sections of the latter than of the former series of plants. The greater rate of sugar utilization by the terminal than the basal sections of the leaves of the plants with nitrate nutrition may be attributed to the synthesis of greater amounts of proteins and also of other nitrogenous substances such as amides and amino acids during and after the assimilation of nitrates.

The rapid utilization of sugars for the synthesis of soluble organic nitrogen compounds, as observed in the ammonium nutrition series, favors the formation of tissues of a high degree of succulence. Root tissues of a high degree of succulence, as obtained under conditions of ammonium nutrition.

are more susceptible to the ravages of parasitic pythiaceous fungi which may accidentally enter nutrient solution than the less succulent tissues of plants with nitrate nutrition.

Total dry organic values are the converse of the moisture content values of the tissues as shown in figure 10. They are, therefore, considerably greater in the nitrate than in the ammonium series of plants. Carbohydrates, representing about 90 per cent. of the constituents of dry organic matter, were produced in greater amounts in the nitrate than in the ammonium series of plants.

### Discussion

Figures 12 to 19 show that the rate of absorption and the mode of assimilation of ammonium nitrogen was quite different from that of nitrate. Ammonium apparently was assimilated more or less completely in the root tissues, the determined products of its assimilation being large amounts of various amino acids and small amounts of glutamine and asparagine. These products accumulated in great quantities in tissues lacking chlorophyll, while in chlorophyllous tissues they were converted readily into proteins.

Nitrate nitrogen was neither absorbed nor assimilated as readily as ammonium. Nitrate, after absorption by the roots, moved through the conducting tissues of the roots and the stem into the leaves. In the basal non-chlorophyllous tissues it occurred in great amounts but as it entered the sub-chlorophyllous tissues of the transitional sub-chlorophyllous sections its concentration decreased and practically disappeared in the chlorophyllous tissues of the blade proper. These data indicate that nitrate was assimilated largely in the green tissues of the leaf. With nitrate nutrition the amounts of soluble organic nitrogen were relatively small except in the stem and in this organ possibly the soluble organic nitrogen was present in large part owing to translocation from the leaves. However, the quantities of protein were comparatively high in both chlorophyllous and non-chlorophyllous tissues, excepting the stem. On a percentage basis the findings indicated a greater rate of *protein* synthesis with nitrate than with ammonium nutrition in all tissues excepting the chlorophyllous ones of the mature C leaves and the nearly fully expanded D leaves of the ammonium series. This may well have been on account of their greater chlorophyll content and possibly greater sugar synthesis which, coupled with a higher rate of ammonium absorption as mentioned before, enabled these leaf sections to store greater quantities of protein.

With respect to the unequal distribution of soluble organic nitrogen in the non-chlorophyllous tissues, resulting either from ammonium or nitrate assimilation, consideration of certain anatomical features of the plant is advantageous. If we attempt to trace the different fractions of soluble

organic nitrogen from the tissues of the roots to those of the leaves of plants with ammonium nutrition we find that the amounts in the basal tissues of leaves decrease gradually from the youngest leaves (group E) to the old leaves (group B) as shown in figure 21-A. Assimilated products move

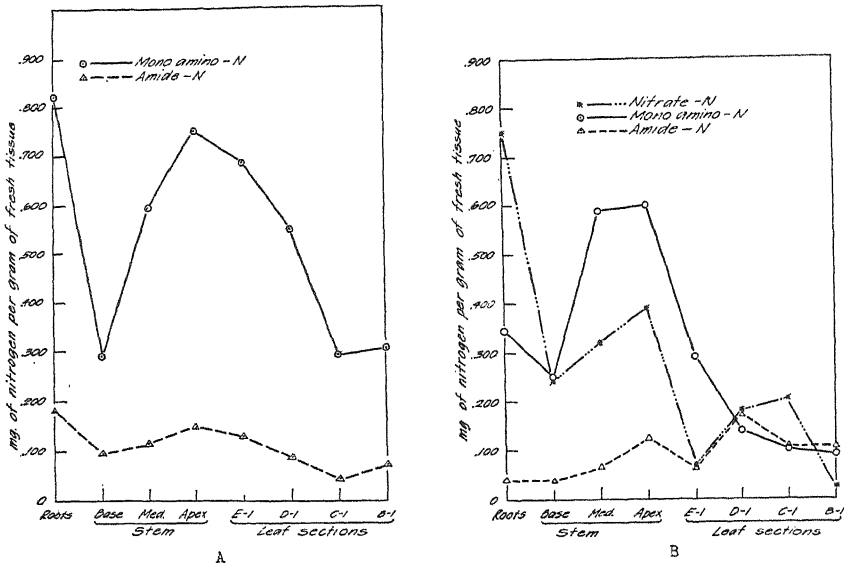


FIG. 21. Trend of distribution of monoamino and amide nitrogen in the non-chlorophyllous tissues of plants with ammonium nutrition (A), and nitrate nutrition (B).

through sieve tubes or conduits of similar physiological function, whereas non-assimilated ones move through tracheae or tracheids. The more recent theories on the movement of organic nitrogen and carbohydrates as expounded by CURTIS (1) and MASON *et al.* (6) claim that such substances may move either downward or upward depending on their location, origin, and ultimate physiological use. Translocation of soluble organic substances to meristematic tissues is generally known to be greater than to tissues which have ceased to grow. Therefore, the presence of greater amounts of soluble organic nitrogen in the younger than in the older tissues is to be expected from the point of view which has been presented.

According to conventional theory the movement of nitrate from roots to leaves is presumably conducted exclusively by the tracheae. In meristematic tissues, such as are in the apex of the stem, the tracheids are in an embryonic state and their capacity for conduction is very low. However, in the mature tissues such as leaves of groups B, C, and D the tracheids are well developed and their capacity for conduction is at a maximum. If we except the basal tissues of leaf group B on account of senility, we find that the amounts of nitrate in the basal leaf tissue of all the other groups are in a descending

scale from mature leaves (C) to young leaves (E) as shown in figures 21-B, which is in harmony with our theoretical expectations. Various other anatomical studies conducted by the senior author (5) have definitely shown that the relative length of individual vascular bundles varies consistently with their position in the stem and the relative length of the internodes. The vascular bundles of the stem that serve the basal tissues of the plant are considerably shorter than those serving the regions toward the apex. With the degeneration of the root primordia in the extreme basal tissue of the stem and the production of roots at higher levels, conduction of water and minerals is diverted by the longer fibrovascular bundles toward the leaves at higher levels on the stem axis. Clearly these observations are also in support of the mode of distribution of nitrate in the basal tissues of the leaves of the different groups as already presented.

Examination of the values in table VII, for ammonium-supplied plants shows that in tissues which have attained complete development but have not yet reached senility, the percentage values of soluble organic and protein nitrogen are practically constant. The actual amounts differ considerably. As an example of this condition there may be examined the percentage values of total soluble and insoluble organic nitrogen of the leaf sections 2 (transitional tissue), 3, 4, and 5 (tip) of mature leaf group C and of active nearly expanded leaf group D. The values for insoluble organic nitrogen range between 71 and 77 per cent., and those for soluble organic from 23 to 29 per cent. The small percentage variations are entirely insignificant when considered from the point of view of absolute variations which, in the case of insoluble organic nitrogen in sections 2, 3, 4, and 5 of leaf groups C and D, range from 0.768 to 2.818 mg. and in that of soluble organic nitrogen from 0.314 to 1.012 mg. With very old or very young leaves similar comparisons may be misleading for reasons stated previously. The sections of the stem showed very great variability, possibly on account of the presence of translocated products and great differences in the age of the tissues of the various sections.

Table VIII, dealing with the nitrate-supplied plants, shows somewhat different results from analogous plant parts. The proportionate values of insoluble organic nitrogen increased gradually from the basal tissue (2) to the tip (5) of the leaves while those of soluble organic nitrogen decreased in the leaves of groups C and D. A similar contrast between the proportional values for inorganic and soluble organic nitrogen showed that the values for both fractions decreased in the same direction. An analysis of the conditions as found in the various tissues shows that the entrance of inorganic (nitrate) was followed by the apparent synthesis of soluble organic nitrogen which was ultimately converted to insoluble organic nitrogen. With processes operating rhythmically, the final or ultimate products of synthesis,

the proteins, would accumulate in greatest amounts in the terminal tissues, and the first products of inorganic nitrogen assimilation, that is, those of soluble organic nitrogen, would be expected to be found in greatest concentration in the basal tissues. The differences in the proportionate distribution of soluble and insoluble organic nitrogen between the different leaf sections of plants with ammonium and nitrate nutrition are apparently caused, as explained above, by differences in their physiological function. The leaves of plants with nitrate nutrition changed inorganic into soluble organic nitrogen besides converting the latter into protein. In contrast, within the limits of nitrogen metabolism, the leaves of the plants of the ammonium series were concerned only with the latter process, as the first one was apparently performed by the roots. The direct inflow of soluble organic nitrogen into the leaves of plants with ammonium nutrition increased the percentage of protein nitrogen in the different sections at approximately the same rate. With nitrate-supplied plants the percentage rate of protein accumulation in the different sections was not constant, because of the direct inflow of unassimilated nitrogen (nitrate) which necessitated conversion first into soluble organic nitrogen and second into proteins.

The data with respect to the mode of assimilation of ammonium and nitrate nitrogen as contrasted in figure 22 show that the ratio between percentages of protein and soluble organic nitrogen is practically 3:1 in the different chlorophyllous leaf sections of the plants of the ammonium series regardless of great variations in the actual weights which are represented as mg./gm. of fresh tissues. Similar conditions are not obtainable in the chlorophyllous sections of the leaves of the plants of the nitrate series, because of the inflow of non-assimilated nitrogen (nitrate) which seemingly interferes with the rhythmical conversion of soluble organic nitrogen to protein. The ratio differences between percentage soluble organic nitrogen and protein of the chlorophyllous sections of plants with ammonium and nitrate nutrition may be employed for differentiating plants grown under the two different types of nutritional conditions.

The factors involved in the assimilation of nitrate have engaged the attention of many workers at different times. SCHIMPER (10) claimed that nitrates become depleted in the green leaves of plants exposed to daylight but that they were retained in those kept in the dark. WARBURG and NEGELEIN (15) and MOORE (7) have supported SCHIMPER's findings. With more extensive studies, which have included a greater number of plant species, information has accumulated showing that different plants behave differently in their mode of nitrate assimilation. According to the information of more recent publications (8), many other plant organs besides leaves are able to assimilate nitrate both in light and in darkness if carbohydrate reserves are adequate.



The rate of nitrate assimilation by the chlorophyllous tissues of pineapple leaves is greater than that of the non-chlorophyllous tissues as indicated by the localization of nitrate and the products of nitrate reduction. Carbohydrates are highly abundant in all non-chlorophyllous tissues. Apparently in the pineapple plant external or other internal factors essential for nitrate assimilation are relatively more favorable in the chlorophyllous tissues.

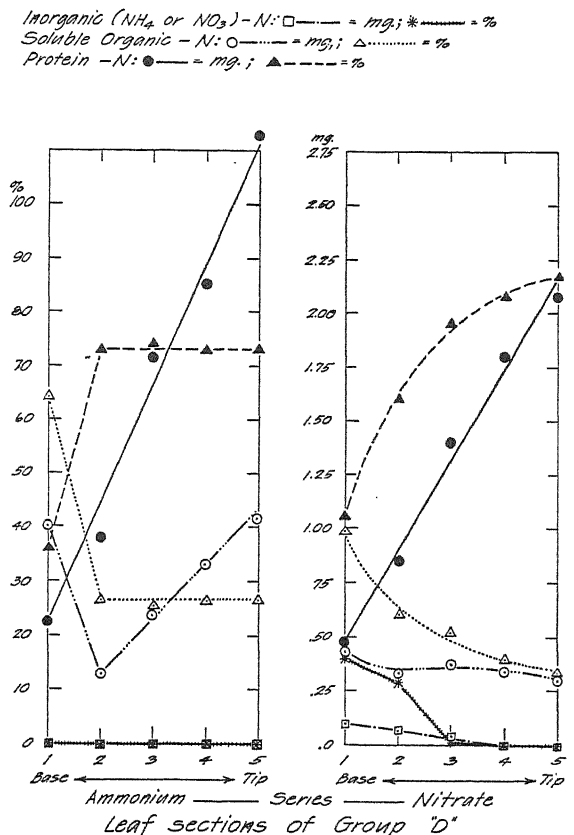


FIG. 22. Distribution of actual weight and percentage values of inorganic, soluble organic, and protein nitrogen in the different sections of the full grown active leaves (group D) of plants grown in ammonium (A) and nitrate (B) solution cultures.

In our earlier publication (13) we have discussed the conditions which, according to certain investigators, favor a higher rate of protein synthesis from nitrate than from ammonium nitrogen.

DISTRIBUTION OF TOTAL AND REDUCING SUGARS AND SUCROSE IN DIFFERENT SECTIONS OF LEAVES, STEMS AND ROOTS OF PINEAPPLE PLANTS GROWN IN SOLUTION CULTURES SUPPLIED WITH EITHER AMMONIUM OR NITRATE SALTS AS SOURCES OF NITROGEN

PLANT TISSUES	AMMONIUM CULTURES			NITRATE CULTURES		
	REDUCING SUGARS	SUCROSE	TOTAL SUGARS	REDUCING SUGARS	SUCROSE	TOTAL SUGARS
<i>Leaves:</i>	%	%	%	%	%	%
<i>Old</i>						
B1 (base).....	1.74	0.39	2.13	2.20	0.42	2.62
B2.....	2.17	0.39	2.56	2.36	0.31	2.67
B3 (tip).....	1.69	0.10	1.79	1.56	0.44	2.00
<i>Mature</i>						
C1 (base).....	2.31	0.29	2.60	2.35	0.43	2.78
C2.....	2.04	0.38	2.42	2.36	0.43	2.79
C3.....	0.99	0.43	1.42	1.46	0.42	1.88
C4.....	0.75	0.76	1.51	1.49	0.54	2.03
C5 (tip).....	0.81	0.77	1.58	1.70	0.55	2.25
<i>Active</i>						
D1 (base).....	2.06	0.05	2.11	2.63	0.42	3.05
D2.....	1.44	0.22	1.66	2.32	0.34	2.66
D3.....	1.40	0.67	2.07	2.08	0.46	2.54
D4.....	1.30	0.89	2.19	2.31	0.67	2.98
D5 (tip).....	1.45	1.05	2.50	1.88	0.71	2.59
<i>Young</i>						
E1 (base).....	1.38	0.28	1.66	1.69	0.45	2.14
E2.....	1.46	0.11	1.57	1.67	0.14	1.81
E3.....	1.49	0.38	1.87	1.95	0.35	2.30
E4 (tip).....	1.80	0.82	2.62	1.23	0.29	1.52
<i>Stem:</i>						
<i>Base</i>						
B-Cortex.....	2.08	0.30	2.28	2.30	0.26	2.56
Pith.....	2.61	0.29	2.90	2.42	0.50	2.92
<i>Middle</i>						
C-Cortex.....	2.61	0.33	2.94	2.86	0.41	3.27
Pith.....	1.87	0.42	2.29	1.98	0.34	2.32
<i>Apex</i>						
D-Cortex.....	1.90	0.26	2.16	2.69	0.63	3.32
Pith.....	1.21	0.61	1.82	1.54	0.68	2.22
<i>Roots</i>						
.....	0.18	0.39	0.57	0.38	0.54	0.92

### Summary

This publication reports the results of experiments conducted under greenhouse conditions with pineapple plants grown in water cultures containing either nitrate or ammonium salts as sources of nitrogen. The objects of the investigations were to obtain data on growth, chlorophyll and moisture content of the different tissues, and distribution of different nitrogenous fractions, the latter to furnish information on the mode of assimilation of ammonium and nitrate nitrogen. The data obtained are presented in tables and figures, and may be summarized briefly as follows:

1. Light conditions were reasonably satisfactory and pH values between 4.2 and 5.5 were maintained as constantly as possible. Nitrogen was not greatly in excess of 28 p.p.m. and under these conditions pineapple plants grew approximately at the same rate when their nitrogen was supplied in the form of either ammonium or nitrate salts.

2. Ammonium nitrogen was absorbed from the nutrient solution at a considerably greater rate than nitrate, and was more rapidly assimilated than the former. The greater rate of ammonium absorption was responsible for the greater quantities of total nitrogen found in plants with ammonium nutrition, and may also account for the higher amounts of chlorophyll and the greater tissue succulence in these plants.

3. Ammonium nitrogen was apparently assimilated in the roots as rapidly as it was absorbed; the immediate products of its assimilation were amino acids, and small quantities of glutamine and asparagine.

4. Nitrate nitrogen was assimilated very slowly, if at all, in the tissues of the roots. Most of it was translocated through the stem and the non-chlorophyllous tissues of the leaf bases presumably to the chlorophyllous tissues. Since little or no nitrate was found there, it would appear that in the chlorophyllous tissues it was converted very rapidly, first into various compounds of soluble organic, especially asparagine and basic amino, and then into protein nitrogen.

5. Plants with ammonium nutrition contained in their non-chlorophyllous tissues comparatively great amounts of soluble organic nitrogen and small quantities of protein. Plants with nitrate nutrition were somewhat different in this respect, the roots especially showed large quantities of protein as compared with those of soluble organic nitrogen.

6. The chlorophyllous sections of the mature C leaves and the active but nearly full grown D leaves of the plants with ammonium nutrition contained greater amounts of both soluble and protein nitrogen than those of the plants with nitrate nutrition. The chlorophyllous sections of leaf group B (old) and E (young) are excluded from this generalization, the former because they contained senile and the latter because they contained immature tissues.

7. The conditions responsible for the differential distribution of inorganic, soluble organic, and protein nitrogen in the corresponding parts of plants with ammonium or nitrate nutrition were caused, at least in part, by the method and rate of assimilation of the two forms of nitrogen.

8. The amounts of reducing sugars and sucrose were in general somewhat greater in the leaf tissues of the plants with nitrate than with ammonium nutrition, indicating a lower rate of carbohydrate utilization in nitrogen assimilation. The quantities of both kinds of sugars were considerably lower in the root tissues of the plants with ammonium than with nitrate nutrition. This condition, coupled with the presence of great amounts of compounds of soluble organic nitrogen, indicates that the sugars of the root tissues of the ammonium-supplied plants were utilized for the synthesis of these compounds from ammonium.

9. Values for the percentage distribution of soluble organic and protein nitrogen in the different sections of the leaves of groups C and D, presented in tables VII and VIII and in figure 22, are more constant for plants receiving the ammonium than the nitrate nutrition. The causes may be ascribed in the case of the plants with ammonium nutrition to the translocated soluble organic nitrogen compounds, which, under a wide variety of conditions, are readily converted to proteins. The irregular distribution of soluble and insoluble organic nitrogen values obtained in the leaves of the plants with nitrate nutrition were associated with the inflow of non-assimilated nitrogen (nitrate) which had first to be converted into compounds of soluble organic nitrogen and then secondly into proteins. Any factor or factors interfering with the first step in assimilation of nitrate might well result in the irregular values of soluble organic and protein nitrogen that were found to occur in the respective leaf sections of the different leaves of the nitrate-supplied plants.

The authors wish to express their appreciation to Dr. G. T. NIGHTINGALE for reading the manuscript.

PINEAPPLE EXPERIMENT STATION  
UNIVERSITY OF HAWAII

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# ELECTRICAL POLARITY AND AUXIN TRANSPORT

W. G. CLARK

(WITH SIX FIGURES)

## Introduction

The polar transport of indole-3-acetic acid (heteroauxin,  $\beta$ -indolyl-acetic acid) in the *Avena* coleoptile has been explained<sup>1</sup> on the basis of electrical polarity (13, 15, 26, 42, 45). The writer (15) has produced evidence showing that the electrical polarity in several plants is in the right direction to explain the basal, polar transport of the negative ion of heteroauxin in plants. He then showed (14, 16) that gravity, light, and applied potentials could reverse the inherent electrical polarity without affecting the polar transport. It was concluded that electrical polarity either has no cause and effect relation to the polarity of heteroauxin transport in the *Avena* coleoptile, or that this relation was not amenable to treatment by the various bioelectric methods utilized. The relation may be real,<sup>2</sup> but more subtle than could be revealed by the electrometric measurements employed.

It was suggested (15) that both bioelectric polarity and polar transport of heteroauxin might be due to polar permeabilities of individual cell membranes in the plant. GUHA (18) described a preferential conductance in the styles of *Narcissus*, *Aesculus*, and *Primula*. A platinum electrode was inserted into the ovary, another into the stigma, and a direct current was passed from a 2-volt source. The "conductivity" of the style was measured by deflections on a galvanometer in series with the plant and electrodes. Current passed more easily from stigma to style than in the reverse direction. Pollination abolished this "unipolar" conductance. GUHA's work is difficult to judge reliably, as platinum electrodes develop large contact potentials very easily and would obscure real effects. Even if this criticism did not hold, GUHA was probably measuring differential polarization capacities rather than preferential conductance (3, 4, 5, 6).

METZNER (30) attempted to demonstrate a "polar conductivity" in the cutinized epidermis of fruits (apple, tomato, *Physalis*, *Clivia*); seed coats (*Pisum*, *Phaseolus*, *Aesculus*); parenchymatous tissue such as discs of the midrib of *Rheum* leaves; root of *Daucus*; and in the algae *Nitella* and *Chara*. He used these objects as rectifiers of an alternating current (A.C.) of 50 cycles, measuring the rectified direct current with a D.C. galvanometer in

<sup>1</sup> WENT and THIMANN'S recent book, *Phytohormones* (45) will be found to contain detailed discussions of most of the procedures and theories mentioned in the following pages.

<sup>2</sup> Sections of *Impatiens* hypocotyls have a bipolar staining capacity. Positively charged dyes are taken up most by basal cut surfaces, and negatively charged ones by apical cut surfaces (15, 42).

series with the calomel half-cells making contact with the tissue studied. No data are given.

The first part of the present paper is concerned with experiments performed to determine whether or not such a thing as unipolar conductance (unidirectional permeability) exists, and if so whether or not it can be correlated with unidirectional or polar transport of heteroauxin in the *Avena* coleoptile. The second part deals with the effect of ether narcosis on electrical polarity. VAN DER WEIJ (40) showed that ether narcosis reversibly abolished polarity, and BONNER (7) pointed out the dependence of polarity on respiration. Hence the effect of ether narcosis on the electrical polarity became a step in the writer's thesis that bioelectrical polarity, as it is ordinarily understood and measured, has no relation to polar transport. The third part of the paper will demonstrate the specific abolishment of polar transport without affecting electrical polarity, protoplasmic streaming, growth, respiration, or turgor. Independence of the mechanisms responsible for lateral transport in tropisms, and longitudinal transport of heteroauxin will be shown to exist.

## Experimentation

### UNIPOLAR CONDUCTANCE

It was shown in an earlier paper (15) that the potential differences (P.D.) of cut sections of coleoptiles were proportional to section lengths. In order to effectively magnify electrical phenomena in sections, it was found very convenient to pile sections one on the other in series. This was done also with the object in view of performing transport tests at the same time as the electrical measurements were made. Twenty-five 5-mm. sections of *Avena* coleoptiles were cut in the VAN DER WEIJ (39) section cutter, placed upright on a tap water agar block  $11 \times 8 \times 1$  mm., a similar agar block being placed on top of the sections. Another twenty-five sections were placed on this block, and so on; so that finally four tiers, each consisting of twenty-five sections, were prepared. Tenth normal KCl agar strips made electrical contact to the top and bottom blocks of this preparation, the N/10 KCl agar leading to N/10 KCl solutions in cups outside of a moist chamber in which the preparation was situated. Calomel-saturated KCl electrodes were placed in these cups, and were used both for applying and measuring P.D. and current passage. Currents of the order used in these experiments were never found to polarize these electrodes. All nonconducting parts were covered with a thin coat of ceresin, and the chamber was darkened.

Several experiments were performed, in which *Pisum* stem-sections and *Avena* sections were used and the inherent P.D. was measured with a Wulf string electrometer (15). The resistance of the setup, with no sections present, was measured by applying a known voltage and measuring the direct



current passage by means of a calibrated galvanometer (sensitivity about 1-mm. deflection for  $10^{-8}$  amperes at 1 meter scale distance, period 5 sec.). Knowing this resistance, the resistance of the sections could be computed by Ohm's law, from the electrometer measurements of the section P.D., and the galvanometer deflections caused by the current flow resulting from the section P.D. The changes in P.D. and resistance were then studied when (1) alternating current, (2) direct current, and (3) ether vapors were applied.

It was found, in *Avena* sections, that small direct currents ( $0.6$  to  $2 \times 10^{-7}$  amp.) caused decreases in resistance essentially equal to those caused by larger D.C. ( $6 \times 10^{-7}$  amp.). This decrease was, in one experiment,  $35,000 \pm 5,000$  ohms for  $0.6 \times 10^{-7}$  amp., as well as for  $6 \times 10^{-7}$  amp. The P.D., however, changed only 1 or 2 millivolts (mv.) when 10 mv. ( $0.6 \times 10^{-7}$  amp.) were applied (fig. 1), while it changed 15 to 20 mv. when 100 mv. ( $6 \times 10^{-7}$  amp.) were applied. In this experiment (fig. 1), a typical case, the morphological bases were, as usual, electropositive to the tips (15). When 10 mv. were applied, in series with the section P.D., the latter dropped 3 mv.; when applied in parallel with the section P.D., no perceptible change resulted. Such a difference is within the limits of individual variation in the determinations. Fifty mv. in series, decreased the inherent P.D. approximately 10 mv., and when in parallel, increased it about 7 mv. Thus in the cases of 3 to 50 mv. applied P.D., the direction of flow of applied current had no real difference in effect on the inherent P.D. But 100 mv., in series, decreased the section P.D. 15 mv., reversing the inherent electrical polarity; and, when in parallel, increased it 20 mv. The time relations of these changes are shown in figure 1. The return (recovery, depolarization) curves show that the processes affected by applied P.D. are reversible. The normal polarity is quickly restored when the applied P.D. is removed. In an earlier paper (16) on applied P.D. and auxin transport, longer periods (5 to 60 or 120 minutes) of current application were used. In those experiments, polarization is more permanent, and depolarization occurs much more slowly. This recalls similar results described by MARSH (29) and by BLINKS (3, 4, 5).

The important conclusion to these observations, so far as the present thesis is concerned, is that the resistance drops when P.D.'s are applied, regardless of the polarity of the applied P.D. This presumably means that the protoplasmic surfaces are altered so that current is carried more easily by ions, *i.e.*, that permeability has increased. The absence of polar effects of the applied P.D. (the differences are within the limits of individual variability) do not confirm the results of MARSH (29) or BLINKS (3, 4, 5), but it is frankly admitted that no attempts were made to study this phase of the problem, and more careful observations may reveal such a characteristic. The sensitivity of the resistance-measuring apparatus was really not high enough to link resistance changes with P.D. changes as quantitatively as could be de-

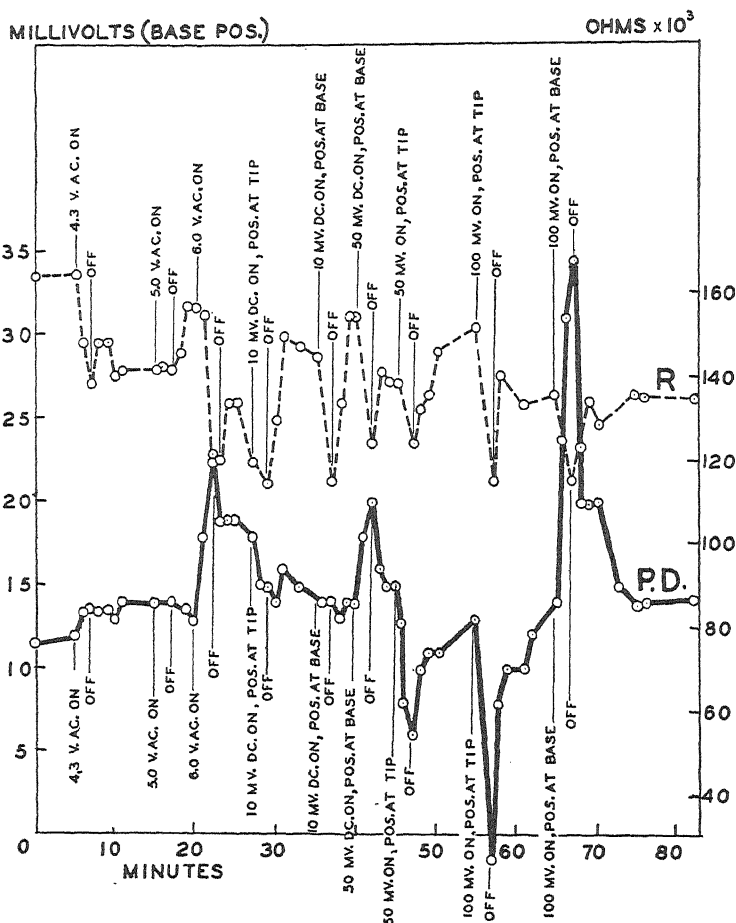


FIG. 1. Effect of applied direct and alternating currents on inherent P.D. and R of *Avena coleoptiles*.

sired, but it is clear enough that the resistance drops just as much with 10 mv. applied P.D. as with 100 mv. (or with 6 volts A.C.).

It would be highly desirable to study more closely the resistance and potential changes with applied P.D., especially since BLINKS (5) and OSTERHOUT (32) have shown that compound membrane potentials may be separated into simpler component P.D.'s by this method; or, in other words, it might be possible to analyze differential membrane permeabilities into simpler components. This may prove to be the only way to attack the electrical phenomena postulated to be linked with polar transport (42), ion accumulation (21), osmotic work performed in intestinal absorption (24) and in the kidney (27), etc., since mere measurements of normal inherent P.D.'s appar-

ently mean little, as has been shown in two former papers by the writer (15, 16), and as will be further proved in this paper.

Figure 2 represents the electrical behavior of coleoptile sections which happened to have had an inverted polarity which was slowly righting itself ("handling reaction," 15). In this experiment, the sections were not piled in tiers. Coleoptiles were decapitated, the primary leaves removed, and the whole coleoptile removed from the rest of the seedling by cutting it just above the coleoptilar node. Forty such whole coleoptiles were placed in parallel, the P.D. between their cut surfaces being measured in the usual fashion.

It is seen in figure 2 that the resistance follows the P.D. Electronegativity decreases as the resistance decreases. The resistance was much lower than in the experiment depicted in figure 1, and hence the measurements were more accurate because galvanometer deflections were greater.

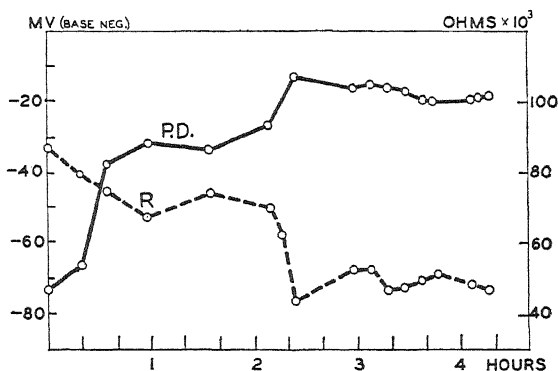


FIG. 2. Variation of inherent P.D. and R of *Avena* coleoptiles with time.

Desiring to see if an A.C. rectifying effect exists in the sections such as postulated by METZNER (30), A.C. (60 cycles) was applied to the sections for 1 to 2 minutes. Figure 3 shows that 3 volts A.C. has little or no effect on the P.D. or resistance (R), whereas 5 v. A.C. reversibly decreases R and increases the electronegativity of the apical surface (cf. effect of ether narcosis). This confirms the results of AMLONG and BÜNNING (1) and BÜNNING (12), who clearly demonstrated a correlation between permeability and bioelectric potentials. The permeability is altered only after a certain threshold of A.C. stimulation is reached (liminal threshold), after which some change in the surface properties of membranes brings about a sudden increase in permeability, and hence a drop in R and a change in P.D. on account of concentration changes and differential ion mobilities.

If one would believe METZNER (30), one would think that the D.C. deflections caused by the applied A.C. meant rectification of the A.C. by the plant tissues. This is an entirely erroneous conclusion, as shown by the P.D.

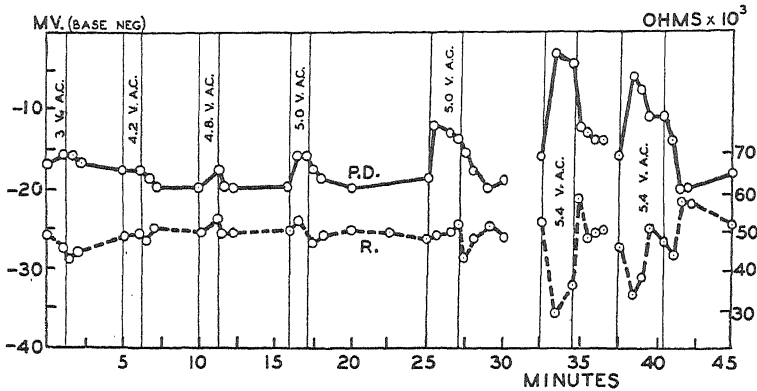


FIG. 3. Effect of applied A.C. on inherent P.D. and R of *Avena* coleoptiles.

measurements. No galvanometer deflection occurs unless the plant P.D. is altered, and hence the galvanometer deflection is caused by stimulation (polarization or permeability change) and not by ohmic rectification of the A.C.

It is seen in figures 1 and 3 that A.C. stimulation increases the negativity of the apical end of the sections (compare also reversible ether narcosis). This may mean that the apex is more easily stimulated or polarized than the base, with subsequent greater increases in permeability or polarization, and with resulting greater apical negativity. Increased permeability (caused by induction shocks) gives rise to negativity of the stimulated portion, as shown by AMLONG and BÜNNING (1).

#### EFFECT OF ETHER NARCOSIS ON ELECTRICAL POLARITY

Ether narcosis irreversibly abolishes electrical polarity if the narcosis results in complete loss of turgor. Figure 4 shows the drop in resistance and P.D. when air, saturated with water vapor, was bubbled through ether and aspirated through the experimental chamber containing the sections which were piled in series as described earlier. After 40 minutes, a residual apical negativity remains which probably represents the geoelectric effect found by BRAUNER and AMLONG (11), and discussed by WENT and THIMANN (45). Figure 4 also shows that 6 v. (A.C.) does not alter the P.D. in the completely narcotized tissue. The D.C. galvanometer, however, showed some deflection. Here, therefore, one might think that METZNER's unipolar conductance is being manifested in a rectification of the A.C. BRAUNER (10) demonstrated a unipolar permeability in certain dead plant tissues (seed coats), but did not determine whether such objects could rectify an A.C. In the narcotized sections, it is more probable that the applied A.C. merely increases permeability a bit more, and hence the R drops a little. The reason

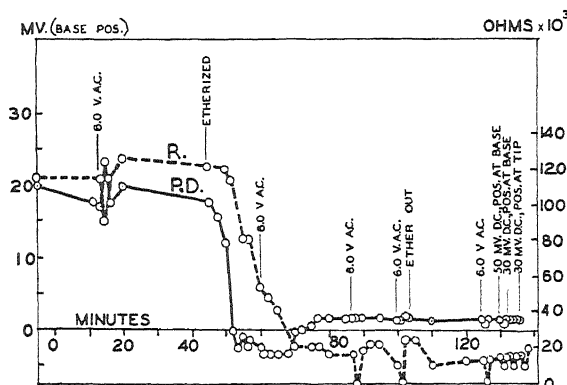


FIG. 4. Effect of irreversible ether narcosis on P.D. and R of *Avena* coleoptiles.

why no further P.D. changes result probably rests on the possibility that the narcosis has irreversibly destroyed selective ion permeability. No ohmic unipolar conductance exists in the narcotized tissue, as shown by equal galvanometer deflections, regardless of the polarity of applied small D.C. (ca.  $10^{-7}$  amp.).

Reversible ether narcosis was obtained in the sections with inverted electrical polarity by passing air through water half saturated with ether at 20° C. and then aspirated through the experimental chamber. As seen in figure 5, the apical negativity was first increased for a brief period, then decreased, just as in complete narcosis. The R changes in the opposite way. The cause of the opposite changes in R in reversible and in irreversible narcosis is not known, as not enough experiments of this nature were performed, but it is well known that small concentrations of ether stimulate such processes as respiration instead of inhibiting them. Reversible narcosis with half-

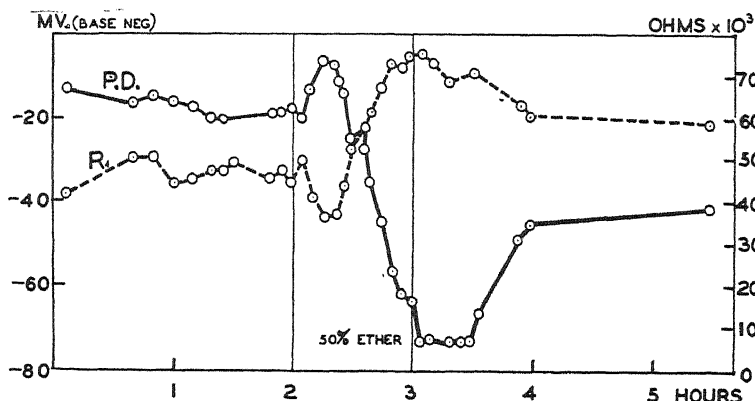


FIG. 5. Effect of reversible ether narcosis on inherent P.D. and R of *Avena* coleoptiles.

saturated ether increases the R of the sections, and decreases their apical negativity. Complete, irreversible narcosis decreases both. Hence the possibility remains that the increased resistance during reversible narcosis signifies a decreased permeability. This decreased permeability may be causally related to decreased polar transport of auxin. VAN DER WEIJ (40), as already mentioned, reversibly abolished polar transport by ether narcosis accomplished by narcotizing coleoptile sections by ether vapors from water half saturated with ether. Approximately this same concentration was used in the reversible narcosis depicted graphically in figure 5. Owing to lack of further experimental data, no further statements can be made at this point, but it is pointed out that here, for the first time, we have been given a clue to the physical properties of the mechanisms responsible for polar transport. It is hoped that further advances in this direction will be made.

#### EFFECT OF CERTAIN LYSINS ON HETEROAUXIN TRANSPORT

SPECIFIC ABOLISHMENT OF POLAR TRANSPORT OF HETEROAUXIN.—It has been shown in the second paper of this series (16) that the longitudinal transport of heteroauxin is independent of measured electrical polarity (apical negativity). This section will show that transport may be specifically abolished without apparently altering any other properties yet investigated. Some of these properties were semi-permeability, cell elongation, protoplasmic streaming, respiration, and electrical polarity. Thus an interesting and promising tool is offered for further investigations into the mechanism responsible for polar transport.

PONDER and MACLEOD (35) showed that the P.D. across a frog skin could be completely abolished by certain lysins, without affecting respiration. It was thought, therefore, that such substances might abolish the electrical polarity of *Avena* coleoptiles without affecting polar transport, since the latter is dependent upon respiration (7, 40).

It was entirely unexpected, therefore, when the results showed just the opposite. The lytic substances abolished transport, but had no effect on electrical polarity, nor, indeed, upon any other observed properties until the concentrations of lysin were high enough to be very toxic.

One way of testing the transportability of various auxins is to see if they will cause curvatures in *Avena* when applied in agar blocks on one side of decapitated coleoptiles. If no curvature ensues, and yet the substance is active in promoting growth, *e.g.*, as in the pea test (19, 45), the substance is not longitudinally transportable. For this reason, the lysins chosen for these experiments, saponin and sodium glycocholate, were applied in agar blocks to the decapitated apices of *Avena* coleoptiles for 3.5 hours, after which a second decapitation was made in which about 1 mm. of the tip was removed. Heteroauxin in agar blocks was then applied unilaterally to the decapitated

stump, the resulting curvatures being recorded 90 minutes after the heteroauxin application. The control curvatures averaged 15°. Table I presents the results. Twelve plants per test were used. (For further details concerning the *Avena* test refer to WENT and THIMANN, 45).

TABLE I

EFFECT OF SAPONIN AND SODIUM GLYCOCHOLATE ON *Avena* TESTS WITH HETEROAUXIN

INFILTRATES IN AGAR BLOCK PLACED ON DECAPITATED COLEOPTILES 3 HR.	CURVATURES**	MEAN ERROR
	<i>degrees</i>	
Tap H <sub>2</sub> O (control) .....	15.0	± 1.8
Sodium glycocholate, 1: 100,000 .....	11.8	± 1.0
Sodium glycocholate, 1: 10,000 .....	10.4	± 0.7
Sodium glycocholate, 1: 1000 .....	12.4	± 1.5
Sodium glycocholate, 1: 50 .....	12.0	± 1.6
Tap H <sub>2</sub> O (control) .....	12.3	± 1.5
Saponin, 1 part saturated: 1000 .....	13.0	± 1.4
Saponin, 1 part saturated : 100 .....	11.3	± 1.8
Saponin, 1 part saturated : 10 .....	15.3	± 1.5

\* The "maximum angle" (WENT and THIMANN, 45) was approximately 20° at the time of these analyses.

Considering the mean errors  $\left(\sqrt{\frac{\sum(d^2)}{n(n-1)}}\right)$ , where  $d$  = deviation from mean, and  $n$  = number of test plants (2), it is clear that no inhibition of *Avena* curvatures was obtained. The conclusion was tentatively reached that the large glucoside molecule, saponin, and the sterol-amino molecule, sodium glycocholate, did not enter the coleoptiles during the pretreatment before heteroauxin was applied.

For this reason, direct transport tests were made in which the coleoptile sections were infiltrated with saponin and sodium glycocholate, with a water-infiltrated lot for controls. Four-mm. sections were cut with the VAN DER WEIJ (39) section cutter, and placed with their hollow centers on the teeth of composition combs, to enable the experimenter to determine their morphological polarity after treatment in various solutions. The combs containing the sections were placed in sodium glycocholate and saponin solutions, and in water, and infiltrated for 2 minutes by releasing negative pressure produced in a desiccator. The combs were then removed, the solutions carefully blown from the hollow centers of the sections, the sections blotted, and six of them placed with their morphological bases downward on tap water agar blocks. Agar blocks containing 0.98 mg. per l. indole-3-acetic acid were placed on the apices. After 2 hours the bottom blocks were removed, cut into 12 smaller blocks, and tested by the *Avena* test, using the double decapitation method which included 3 hr. between decapitations and 90 min. between time of putting the agar blocks on the test plants and photographing them (37).

TABLE II

EFFECT OF SODIUM GLYCOCHOLATE AND SAPONIN ON HETEROAUXIN TRANSPORT IN  
COLEOPTILE SECTIONS

CONCENTRATION TEST (0.073 MG./L.) ..... 8.4°  
7.3

Av. 7.8

7.8° = 1/40 STOCK SOL. (2.98 MG./L.), HENCE STOCK =  $7.8 \times 40 = 312^\circ$

TOP BLOCKS IN TRANSPORT TESTS =  $\frac{1}{3}$  STOCK =  $312/3 = 104^\circ$

TRANSPORT TIME 2 HR.

6 4-MM. SECTIONS PER TEST

INFILTRATE IN SECTIONS	CURVATURE (AUXIN TRANSPORTED INTO BOTTOM BLOCKS)	MEAN ERROR	PROTO- PLASMIC STREAMING	APPEAR- ANCE OF SECTIONS
	<i>degrees</i>			
Sodium glycocholate, 1: 100 .....	None	.....	-	Flaccid
Sodium glycocholate, 1: 500 .....	None	.....	+	Turgid
Saponin, 1 part saturated: 20 ...	10.4	$\pm 1.4$	-	Turgid
Saponin, 1 part saturated: 100 ...	11.1	$\pm 1.3$	+	Turgid
H <sub>2</sub> O .....	10.2	$\pm 1.1$	+	Turgid

Table II shows the results. One column of this table shows the result of a microscopic examination for protoplasmic streaming in the sections after the transport tests had been made. The sections showed protoplasmic streaming when placed intact in a drop of water on a slide, or when cut into two halves with a razor blade and examined under water with white light and under 900 magnifications. Another column of the table describes the macroscopic condition of the sections.

The following conclusions can be formulated from the results given in table II: (1) Sodium glycocholate is toxic in a concentration of 1:100. It is not toxic in 1:500 for the time used. The criteria for toxicity were taken to be cessation of protoplasmic streaming, and the occurrence of flaccidity caused by loss of semipermeability and turgor; (2) Sodium glycocholate in nontoxic concentrations completely abolishes heteroauxin transport. This is not due to cessation of protoplasmic streaming; (3) Saponin, in concentration of 0.05-saturated, stopped protoplasmic streaming but had no effect on transport. This is very interesting, in view of the fact that WENT (41) and BOTTELIER (9) suggested that protoplasmic streaming may limit transport. SCHUMACHER (36), however, has shown that the transport of fluorescein in stem hairs of *Cucurbita pepo* is independent of protoplasmic streaming. Additional data concerning this relationship are needed. Saponin in higher dilution had no effect on heteroauxin transport, turgor, or protoplasmic streaming.

It is conceivable that the effect of sodium glycocholate may not be limited to the membrane mechanisms responsible for polar transport. In higher dilution (1:500) it does not obviously abolish semipermeability, as judged



by the retention of turgor; nor does it affect protoplasmic streaming. It might, however, inhibit some other mechanism which limits transport, for example, cell oxidations, and presumably, therefore, the sources of energy for the performance of concentration work in polar transport (*cf.* HOAGLAND and BROYER, 21, HUF, 22, 23, and VAN DER WEIJ, 40), who show that concentration work depends on respiratory metabolism of the living membrane). BONNER (7) has shown that growth by cell elongation in the *Avena* coleoptile is limited by respiration. If, therefore, sodium glycocholate affects respiration, it should affect growth. Since, however, growth in intact *Avena* coleoptiles is limited by transport,<sup>3</sup> the effect of sodium glycocholate on growth must, on first thought, be studied by means of the pea test (34, 43, 45). In this test, polar transport does not limit growth. Hence the effects of sodium glycocholate and saponin on heteroauxin curvatures in the pea test were investigated. Etiolated *Pisum* seedlings were used about 7 days after soaking and planting. The first leaves were removed, and the apical end of the stem was split longitudinally for about 5 cm. The stem was then cut off just below the split part, and the split stems put into the solutions to be tested. Heteroauxin enters the intact sides of the split halves more than on the cut sides (34), increasing growth there, so that the two halves curl inward. The extent of this curvature is proportional to the activity of the heteroauxin on cell elongation.

The split pea stems were put into heteroauxin solutions containing saponin and sodium glycocholate. The curvatures were photographed after 3, 6, and 11 hours, the resulting curvatures being measured and plotted in figure 6. The concentration of heteroauxin used was 1.96 mg. per liter. Seven tests per solution were made.

It is seen that both sodium glycocholate and saponin in concentrations which do not cause a decrease in turgor, not only do not inhibit growth, but actually increase the final curvatures between 50 and 60 per cent. higher than the heteroauxin controls. Sodium glycocholate alone had a very small activity on the pea test, and saponin alone had none. The method of the facilitation of heteroauxin activity by these lytic substances is not yet known.

The important conclusion for the purposes of this discussion is, however, that apparently nontoxic concentrations of lysins do not inhibit the action of heteroauxin on plant growth, while one of them (sodium glycocholate) completely abolishes polar transport. This conclusion has been justified by repetition of the above experiments, using sodium glycocholate in concentrations as small as 1:100,000. The following experiment illustrates this justification:

<sup>3</sup> See section below in which the effect of sodium glycocholate on the growth of cut sections of coleoptiles shows that transport does not limit the growth of cut sections. This does not agree with BONNER'S statement that transport in sections limits growth, as shown by inhibition by lack of oxygen.

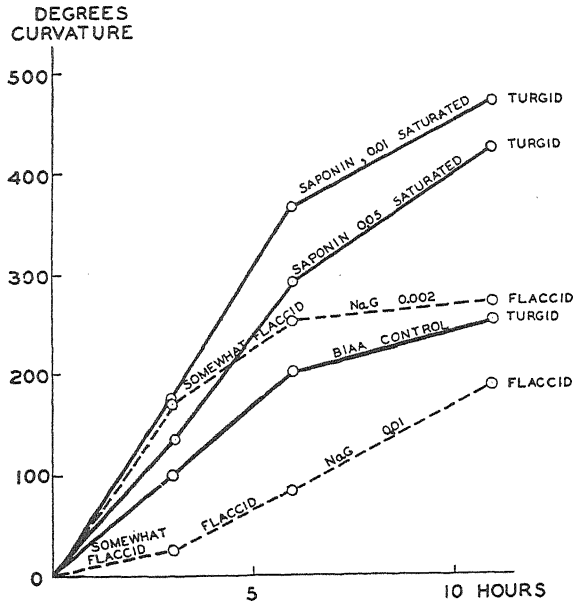


FIG. 6. Effect of sodium glycocholate and saponin on pea stems tested with heteroauxin (BIAA).

Four-mm. coleoptile sections were infiltrated, blotted, and a 2-hour transport test was performed with heteroauxin of a concentration of 356° in the apical blocks of six upright sections. The basal blocks were tested by the *Avena* test, using the double decapitation method. The sections were infiltrated with aqueous sodium glycocholate solutions of the concentrations indicated in table III.

The conclusion is that sodium glycocholate abolishes transport in very high dilution (dilutions higher than 1 : 100,000 have not as yet been tested), although protoplasmic streaming and semipermeability are unaffected.

TABLE III  
EFFECT OF SODIUM GLYCOCHOLATE ON HETEROAUXIN TRANSPORT IN COLEOPTILE SECTIONS

INFILTRATE IN SECTIONS	ANALYSIS OF BOTTOM BLOCK (CURVATURE)	MEAN ERROR	PROTO- PLASMIC STREAMING	APPEAR- ANCE
	<i>degrees</i>			
H <sub>2</sub> O .....	11.4	± 1.4	+	Turgid
Sodium glycocholate, 1: 100,000.....	None	.....	+	"
Sodium glycocholate, 1: 10,000.....	"	.....	+	"
Sodium glycocholate, 1: 5000.....	"	.....	+	"
Sodium glycocholate, 1: 1000.....	"	.....	-	"
Sodium glycocholate, 1: 500.....	"	.....	-	Flaccid

As stated above, PONDER and MACLEOD (35) found that respiration was unaffected in frog skin, although the P.D. was abolished by 1:100 saponin and sodium glycocholate. It was shown above that heteroauxin transport is presumably specifically abolished.

The following experiment shows the effect of sodium glycocholate on the electrical polarity of coleoptile sections from the same lot that was infiltrated for the transport tests. The sections were placed in tiers to give series summation of P.D. Two tiers of ten sections each were piled up, and the P.D. between morphological tips and bases measured with the electrometer. Normal polarity existed, since the bases were electro-positive. Table IV shows the results of measurements 2 hours after setting up (about the same time that the transport tests were ended).

TABLE IV

EFFECT OF SODIUM GLYCOCHOLATE ON ELECTRICAL POLARITY OF COLEOPTILE SECTIONS

INFILTRATE IN SECTIONS	P.D. (BASE ELEC- TROPOSITIVE)	PROTOPLASMIC STREAMING	APPEARANCE
	<i>mv</i>		
H <sub>2</sub> O .....	10	+	Turgid
Sodium glycocholate, 1: 100,000 .....	10	+	"
Sodium glycocholate, 1: 10,000 .....	10	+	"
Sodium glycocholate, 1: 5000 .....	5	+	Less turgid
Sodium glycocholate, 1: 1000 .....	0	-	Flaccid
Sodium glycocholate, 1: 500 .....	0	-	"

The unexpected conclusion was reached that although 1:100,000 down to 1:5000 sodium glycocholate completely abolishes heteroauxin transport during the 2-hour transport test, it has no effect on electrical polarity (or protoplasmic streaming and semipermeability).

Pea tests confirmed the earlier observation that sodium glycocholate increases the activity of heteroauxin. In this test, unlike the former, the split pea stems were first infiltrated with the sodium glycocholate solutions before being placed in the sodium glycocholate-heteroauxin solutions. Such infiltration of the pea stems thus makes their physiological state more comparable with that of the infiltrated coleoptile sections. The pea curvatures were measured after 12 and 24 hours. The concentration of heteroauxin used was 0.73 mg. per liter. Ten tests were made in each solution. Table V shows the results:

The same facilitation by sodium glycocholate (NaG) of the final curvature reached in heteroauxin solutions is thus confirmed. NaG 1:5000, again gave an increase over the heteroauxin control, in this case an increase of 60 per cent. The control, NaG 1:1000 gave a heteroauxin-like curvature, but

TABLE V

EFFECT OF SODIUM GLYCOCHOLATE ON PEA TEST CURVATURES IN HETEROAUXIN

SOLUTION	EFFECT ON PEA STEMS			
	DEGREES CURVATURE (WITH MEAN ERROR) AFTER 12 HOURS	CONDITION	DEGREES CURVATURE (WITH MEAN ERROR) AFTER 24 HOURS	CONDITION
Heteroauxin-H <sub>2</sub> O (control) .....	<i>degrees</i> 150° ± 11°	Turgid	<i>degrees</i> 173° ± 11°	Turgid
Heteroauxin-Sodium glycocholate, 1: 100,000 .....	164 ± 13	"	224 ± 13	"
Heteroauxin-Sodium glycocholate, 1: 10,000 .....	169 ± 19	"	211 ± 24	"
Heteroauxin-Sodium glycocholate, 1: 5000 .....	269 ± 14	"	290 ± 14	"
Heteroauxin-Sodium glycocholate, 1: 1000 .....	194 ± 15	Flaccid	← (Too flaccid to measure) →	
Heteroauxin-Sodium glycocholate, 1: 500 .....	131 ± 14	"	" " "	"
Sodium glycocholate (control), 1: 5000 .....	- 62 ± 18	Turgid	Negative*	Turgid
Sodium glycocholate (control), 1: 1000 .....	70 ± 17	← (Too flaccid to measure) →		

\* Negative curvature signifies no heteroauxin effect.

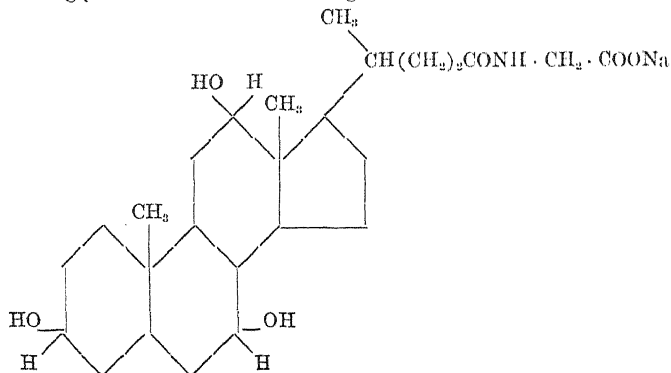
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the sections were completely flaccid. NaG 1:5000 gave no curvature, thus impurities in the NaG do not produce the effect.<sup>4</sup>

It was shown that NaG does not inhibit growth in the pea test. To make the growth test more complete, growth of *Avena* coleoptile sections, infiltrated with NaG and put into heteroauxin solutions with NaG, were measured.

Approximately 4-mm. sections were cut with the VAN DER WELJ section cutter, placed on teeth of combs, infiltrated with 1:100,000 NaG, and put in

<sup>4</sup> Sodium glycocholate has the following structure:



0.74 mg. per liter heteroauxin solutions. Twenty-two sections (2 per coleoptile) were used for each test. Measurements were made 12 and 30 hours after the start of the experiment. Table VI presents the results, expressed in millimeters.

TABLE VI

EFFECT OF SODIUM GLYCOCHOLATE ON GROWTH OF COLEOPTILE SECTIONS IN  
HETEROAUXIN (0.74 MG./L.)

INFILTRATE IN SECTIONS	SECTION LENGTHS				
	0 HOURS	12 HOURS	PERCENTAGE INCREASE IN 12 HOURS	24 HOURS	PERCENTAGE INCREASE IN 24 HOURS
	<i>mm.</i>	<i>mm.</i>	<i>%</i>	<i>mm.</i>	<i>%</i>
H <sub>2</sub> O .....	4.35 ± 0.01	4.70 ± 0.1	7.9	5.12 ± 0.6	17.7
Sodium glyco- cholate .....	4.35 ± 0.01	4.74 ± 0.2	9.0	5.09 ± 0.6	17.0

After 30 hours all sections used in the above experiment showed protoplasmic streaming, and were turgid. It can be concluded that the growth of the *Avena* coleoptile is not affected by NaG, which completely abolishes heteroauxin transport. At first, one might ask how coleoptile growth responds to heteroauxin when transport is abolished? WENT (44) has pointed out that polar transport is like ion accumulation in barley roots, potato tuber discs, etc. (21), in that the polarity is the result of an excess basal transport over apical transport. Over long periods of time, therefore, polarity will not be limiting, especially in the high concentrations of heteroauxin used. The effect of polarity of transport (and entrance of heteroauxin into the sections) on section growth would probably be observed only in the first few hours, where the velocity of transport of heteroauxin into the control sections would result in a much higher rate than in the sections in which polarity is abolished by NaG treatment. Ordinary diffusion and apical transport ultimately lets enough heteroauxin into the NaG-treated sections to permit growth to overtake the growth of the control sections. Whether or not the above suggestion is correct, the important fact is that growth by cell elongation is unaffected in *Avena* coleoptiles treated with NaG in a concentration which abolishes polar heteroauxin transport.

It can be predicted, *a priori*, that NaG will not inhibit respiration, since growth is unaffected, and since BONNER (7) has shown that growth by cell elongation depends on respiration. This prediction was realized by respiration measurements of *Avena* coleoptile sections.

The WARBURG manometric technique (38) was employed in making the respiration measurements. Twenty 4-mm. coleoptile sections were placed in

3 cc. of MACILVANE'S phosphate buffer (0.005 M) at pH 6 in vessels of approximately 17-cc. volume (vessel constants known). The central wells in the vessels were filled with 0.3 cc. 25 per cent. KOH. The constants were corrected for the volume of the sections. The vessels were shaken at moderate speed in a constant temperature bath at 25° C. in the dark; and readings on the manometers were taken every 30 minutes for 2 hours. One vessel contained sections infiltrated with tap water, another sections infiltrated with 1:100,000 NaG, and the third vessel acted as the barometer. The sections consumed an average of 9.9 mm.<sup>3</sup> O<sub>2</sub> each 30 minutes in the buffer control; and 9.7 in the NaG-treated. The QO<sub>2</sub> (mm.<sup>3</sup> per section per hr., at standard temperature and pressure) was 1.21 for the control and 1.22 for the sections treated with NaG. These figures are of the order found by BONNER (7) for *Avena* coleoptile respiration.

The results show that respiration of coleoptile sections is unaffected by NaG of a concentration which abolishes heteroauxin transport. As stated above, this finding would at first sight disagree with BONNER'S (7) statement that transport of heteroauxin into coleoptile sections depends upon respiration. BONNER'S conclusions were based on experiments in which sections were placed in an O<sub>2</sub>-free environment. VAN DER WEIJ (40) has also shown that polar transport is reversibly abolished by ether narcosis. The probable explanation is, however, that lack of oxygen and narcosis affect respiratory mechanisms limiting the polarity phenomenon, while sodium glycocholate merely removes the *connecting link* between respiration and transport. To prove that respiration does not limit transport, one would have to abolish respiration without affecting transport, and this has been impossible, so far as the writer is aware. It is conceivable that respiration in certain plant cells which concentrate ions (21) may also be separated from the accumulation mechanism by treatment with similar lysins (this point will be dealt with in a later section of this paper). In other words, respiration may continue without polar transport or ion-accumulation (?), but if respiration is inhibited, both (1) transport (40) and (2) ion accumulation (21) are inhibited.

Thus for the first time, a method has been found which specifically abolishes polar transport. Further experimentation on other physiological processes which are altered during this specific inhibition (*e.g.*, polarization capacity) should reveal the mechanism of polar transport in more detail.

EFFECT OF NaG ON LATERAL HETEROAUXIN TRANSPORT.—If lateral transport is caused by membrane potentials, as is claimed by many plant hormone workers (see introduction to first paper of this series, 15), it seemed possible that sodium glycocholate would abolish lateral transport of heteroauxin, and therefore photo- and geotropisms. This conception was based on the observation of PONDER and MACLEOD (35) that sodium glycocholate and saponin abolished frog skin potentials.

*Phototropism.*—*Avena* coleoptiles were removed from etiolated seedlings. One-half of the undecapitated coleoptiles were removed from the primary leaves, the other half was left with leaves in place. One-half of each lot was infiltrated with water, the other half with 1:100,000 sodium glycocholate. The coleoptiles were then placed upright, and exposed to a 60-watt gas-filled incandescent lamp (tungsten filament) at 4 meters for 2 hr. at 25° C. (Assuming a mean horizontal candle power of 100 for the 60-watt lamp, the meter-candle-second value becomes approximately 45,000 MCS. The coleoptiles without leaves curve at a faster rate than the ones with leaves, but the final curvature of both sets was the same. Photographs were made of the curvatures, and the measurements and mean error are shown in table VII.

*Geotropism.*—Coleoptiles prepared as described previously were placed on their sides for 3 hours (25° C. in the dark), after which photographs were taken, and the curvatures measured. Twenty-four coleoptiles were infiltrated with water, and an equal number with 1:100,000 sodium glycocholate. The resulting curvatures are given in table VII.

TABLE VII

EFFECT OF SODIUM GLYCOCHOLATE ON LATERAL TRANSPORT OF HETEROAUXIN IN COLEOPTILES

INFILTRATE	PHOTOTROPISM (24 COLEOPTILES, 45,000 MCS)
	CURVATURE degrees
Water .....	17.8 ± 3.1
Sodium glycocholate, 1:100,000 .....	20.1 ± 3.3
	GEOTROPISM (24 COLEOPTILES, 3 HR. PRESENTATION)
Water .....	43.8 ± 5.3
Sodium glycocholate, 1:100,000 .....	42.7 ± 6.5

It is quite obvious that sodium glycocholate, in a concentration which entirely abolishes longitudinal transport, has no effect on photo- or geotropism. VAN OVERBEEK (33) has shown that auxin-a destruction by light may partially account for phototropism in *Avena*, but lateral transport of auxin is the most important factor in photo- as well as geotropism, as has been quantitatively proved by WENT (41), DOLK (17), BOYSEN-JENSEN (8), and others.

It has been stated on a former occasion (16) that lateral and longitudinal transport of auxin in plants is caused by entirely different mechanisms. This would be a *a priori* predicted, since lateral transport is induced, and may occur toward either side of stems or roots; while longitudinal polar transport

is inherent, and may not be easily reversed, if indeed at all. The difference between the two types of transport was also demonstrated by the facts that gravity, light, and an electric field affect lateral,<sup>5</sup> but not longitudinal, transport.

The difference is further indicated by the unequal effects of sodium glycocholate on the two types of transport. Furthermore, MICHENER (31) showed that geotropism may no longer be induced in *Pisum* seedlings after treatment with ethylene.<sup>6</sup>

VAN DER LAAN (28) claimed that lateral transport of auxin in horizontally placed *Vicia faba* epicotyls is reversed in ethylene, i.e., that the auxin is displaced to the upper side of the stem. He also showed that ethylene has no effect on longitudinal auxin transport in *Avena* coleoptiles. In general, experiments with ethylene have shown that lateral transport is inhibited or reversed, but that longitudinal transport is unaffected, thus the exact reverse of the sodium glycocholate effect.

The sodium glycocholate effect is not one on electrical polarity, as is shown by the experimental evidence presented in this paper. Evidence from other sources (15) has shown a close, if not causal, relationship between lateral displacement of auxin and geoelectric P.D. The irreversible narcosis experiments described in an earlier part of this paper showed that geoelectric P.D. probably constitutes some portion of normal electrical polarity. On such evidence it could be predicted beforehand that sodium glycocholate would not affect lateral displacement of heteroauxin. To what, then, is the effect of sodium glycocholate on longitudinal heteroauxin transport due?

Sodium glycocholate is a lysin, and is highly lipophilic. In high concentration, it probably promotes the solution of lecithin and various lipoids from the cell membranes, and finally completely destroys the membranes. In higher dilutions, however, it may merely compete with the surface-active heteroauxin for surfaces. If heteroauxin is transported either by (1) surface-spreading (15), or by (2) adsorption to a larger, cataphoretically transported negative particle, this surface competition would abolish transport. Moreover, as PONDER and MACLEOD (35) pointed out, if P.D.'s are abolished by lysins such as sodium glycocholate and saponin, an electrical transport of the heteroauxin anion would be abolished. As yet, no evidence exists which indicates such an electrical transport, but the methods of measurements so far used may be entirely insufficient to demonstrate electrical properties of individual membranes possibly responsible for transport polarity. As pointed out in the section on applied P.D. and unipolar conductance, it is

<sup>5</sup> Lateral transport of auxin in electrotopism has not as yet been directly measured.

<sup>6</sup> Ethylene increases heteroauxin destruction in *Pisum*, but not enough to completely inhibit growth. If growth can occur, geotropism should occur if lateral transport may take place (31).



hoped that polarization-capacity experiments will reveal such properties, especially when studied during reversible narcosis. Further, a homologous series of surface-active substance (*e.g.*, the phenyl or indole series of auxins) might show an effect on longitudinal transport of auxin-a or heteroauxin, the effect being greater as higher members of the series are used. This would be interpreted as an increasing competition for surfaces. Polarization-capacity studies made during such treatments might reveal further functional or structural changes in cell membranes due to the action of the surface-active homologues.

#### EFFECT OF NaG ON BROMIDE ACCUMULATION BY BARLEY ROOTS

This section deals with a few experiments performed in the hope that it might be shown that sodium glycocholate abolished ion accumulation in barley roots, a phenomenon shown by HOAGLAND and BROYER (21) to be dependent upon oxidations, just as is polar transport. Furthermore, WENT (44) indicated a similarity between the two, and the present paper has already suggested similarities. It will be shown, however, that there is no effect of sodium glycocholate on bromide accumulation in roots.

Barley seeds were soaked in tap water 24 hours, planted in chloride-free sand on wire mesh, germinated in tap water in the dark room at 25° C. and 90 per cent. relative humidity until about a week old, after which they were transferred into the diffuse light of the laboratory for 2 hours, and then into the green house. HOAGLAND and BROYER (21) substituted chloride-poor solution for the tap water. After the root system was about 2 weeks old, the roots were excised, washed with distilled water twice, weighed, dried at 100° C. for an hour, and reweighed. The difference between wet and dry weights was assumed to be proportional to the sap content. The dried roots were digested in Na<sub>2</sub>O (0.5 gm. per gm. wet weight), the digest evaporated on a hot plate, neutralized to congo-red paper, filtered, the residue washed several times with hot water, and the collected filtrates titrated electrometrically with a silver bromide electrode (against a calomel electrode, using a KNO<sub>3</sub>-agar bridge) for total bromide (20). Excised roots were placed in flasks containing chloride-free and salt-poor solutions (21) containing sodium glycocholate, 1:100,000, 1:10,000, and 1:5000, and the controls containing no sodium glycocholate. Another lot was run in which this setup was duplicated, but in addition to which NaBr (0.005 M) was added. The flasks were vigorously aerated by bubbling oxygen through sintered glass. After 12 to 24 or 48 hours, the roots were removed, washed twice in distilled water, and analyzed for bromide. No chlorides were present in the controls.

The electrometric endpoints were highly satisfactory, and easily obtained. Sodium glycocholate had absolutely no effect on bromide accumulation by these roots. Table VIII shows two typical cases.

TABLE VIII

EFFECT OF SODIUM GLYCOCHOLATE ON BROMIDE ACCUMULATION BY BARLEY ROOTS

EXPERIMENT	MILLIEQUIVALENTS BR- PER LITER			
	A NaBr IN SAP	B NaBr IN SOLUTION	C AMOUNT ACCUMULATED (A-Ac*)-B	D ACCUMULA- TION RATIO (A-Ac*)/B
6/17/37				
Accumulation time (12 hr.)				
Control .....	0.541(Cl-?)	0	.....	.....
NaBr .....	11.82	4.75	6.53	2.38
NaBr + Sodium glyco- cholate (1:10 <sup>5</sup> ) .....	13.20	4.75	7.91	2.66
6/21/37				
Accumulation time (15 hr.)				
Control .....	0.114(Cl-?)	0	.....	.....
NaBr .....	15.30	5.00	10.20	3.04
NaBr + Sodium glyco- cholate (1:10 <sup>4</sup> ) .....	16.9	5.00	11.80	3.36

\* Ac = A control.

A few experiments on bromide accumulation in *Nitella* showed that 1:100,000 sodium glycocholate was toxic, and allowed electrolytes already present in the sap to escape.

It must be concluded that ion accumulation in roots, and polar transport of auxins are not brought about by the same types of mechanisms. It was therefore conjectured that only surface-active ions will display polar transport, or be prevented from accumulating. A few experiments were then performed in which it was attempted to demonstrate accumulation of heteroauxin by *Nitella* cells, using the FeCl<sub>2</sub> colorimetric test for heteroauxin (FeCl<sub>2</sub> and concentrated H<sub>2</sub>SO<sub>4</sub> are boiled with heteroauxin. 1:10<sup>6</sup> will give a pink color) in expressed sap of cells placed in heteroauxin solutions (tap water) from 1 to 50 mg. per liter, which gave very clear tests by this colorimetric method, both before and after the experiments. The test was, however, much weaker after a run than before, and no test was obtained inside the cells (expressed sap). It was concluded that either (1) heteroauxin is completely destroyed in the sap, once it penetrates, or (2) that it is present in concentrations too small to be detected by this method, and therefore is not accumulated. The cells were probably quite healthy and normal, as shown by the fact that they contained nearly 0.1 N in chlorides

(as tested by electrometric titration with a chloride electrode), while unhealthy cells frequently lose their accumulated ions (21).

The writer suggests further experiments on the effect of lysins on accumulation of organic and inorganic electrolytes and nonelectrolytes, including surface-active ones.

### Summary

1. Sections of *Pisum* stems and of *Avena* coleoptiles are reversibly polarized by applied direct currents beyond certain thresholds of applied current. The normal electrical polarity may be reversibly increased or decreased, or reversed by these currents.

2. Apical negativity (normal polarity) is increased, within limits, on stimulation by alternating currents, but only after certain thresholds of applied currents are reached.

3. *Avena* coleoptiles cannot act as ohmic rectifiers of an alternating current, as claimed by METZNER (30) for many plant tissues, nor do they display a unipolar resistance to a direct current. This means that a possible asymmetry in membrane permeability theoretically linked with polar transport (accumulation, secretion?) cannot be analyzed by simple unipolar or rectification measurements.

4. The apical negativity of *Avena* coleoptile sections is reversibly decreased by ether narcosis, and the P.D. drops to that caused by gravity when narcosis is irreversible.

5. The resistance of *Avena* coleoptiles reversibly decreases on passage of small direct currents and alternating currents.

6. The resistance changes follow the inherent P.D. changes, but in the opposite sense, *i.e.*, when apical negativity increases, the ohmic resistance decreases. An exception occurs in complete irreversible ether narcosis, where both resistance and P.D. decrease.

7. Polar heteroauxin transport in *Avena* coleoptiles may be specifically abolished with 1 part of sodium glycocholate in 100,000 parts of water, without there being any change in *electrical polarity*, respiration, semi-permeability, growth by cell elongation, or protoplasmic streaming.

8. Lateral and longitudinal transport of auxin-a in plants are caused by entirely different mechanisms, as shown by the different effects of light, gravity, applied potential differences, ethylene (31), and sodium glycocholate.

9. The apparent relation of auxin transport in plants to ion accumulation by barley roots, as suggested by WENT (44), is shown to be somewhat questionable, as sodium glycocholate abolishes the former but has no effect on the latter.

10. Statements 1 to 7 lend additional support to former statements made by the writer (15, 16) that electrical polarity, expressed in terms of

inherent potential differences (unipolar conductance, or rectification of alternating current) has no apparent causal relation to polar auxin transport in plants.

CALIFORNIA INSTITUTE OF TECHNOLOGY  
PASADENA, CALIFORNIA

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# CONDUCTIVITY MEASUREMENTS OF PLANT SAP<sup>1</sup>

GLENN A. GREATHOUSE

(WITH TWO FIGURES)

## Introduction

The plant physiologist recognizes more and more the importance of the application of physics and physical chemistry to fundamental plant problems, realizing that a mastery of botany, physics, chemistry, and mathematics will aid in the understanding of the living cell.

Conductivity measurements have been widely used in the study of plant problems in recent years (7, 8, 12, 14, 15, 17, 25, 27, 28, 32). A critical examination of these articles indicates the desirability of reviewing the basic principles of conductivity measurements and of pointing out the various factors that influence the conductivity value of plant sap. This paper will discuss the application and limitation of the measurement of electrical conductivity of plant saps, with particular reference to cold-hardened and unhardened plant tissues.

## Experimental determination of conductivity

The conductivity of an electrolyte is measured in terms of the resistance of the solution between two electrodes, and it is the reciprocal of the resistance. The resistance may be measured by the alternating current Wheatstone bridge method as employed by Kohlrausch or by modification of this method. For the theory of bridge measurements the reader is referred to standard textbooks of physics and physical chemistry.

The essential features of an apparatus for measuring the resistance of an electrolyte by the Kohlrausch method are indicated in figure 1. In this diagram  $C$  is the electrolytic cell,  $R$  is a variable resistance,  $X$  is the condenser,  $E$  is a source of alternating current,  $T$  is the current detector,  $a$  and  $b$  the ends of the bridge, and  $d$  the movable contact.  $R_1$  is a resistance coil (the resistance of each coil is usually  $4\frac{1}{2}$  times that of the wire, making the latter 0.1 of the total resistance of the ratio arms) and  $K$  is a switch to short circuit the coil  $R_1$ .

Kohlrausch's classical method has had the benefit of numerous improvements in recent years. The researches of WASHBURN (38, 40, 41) have been

<sup>1</sup> Measurements of electrical conductivity have many applications in physiological investigations. The importance of knowing the underlying principles involved in such measurements as well as the limitations of application to plant physiological problems cannot be overemphasized. In this report prepared by Dr. GLENN A. GREATHOUSE for the Physical Methods Committee measurements of conductivity and their applications are discussed. This report, together with its bibliography, should be helpful to those working in this field of research.—Earl S. Johnston, *Chairman*.

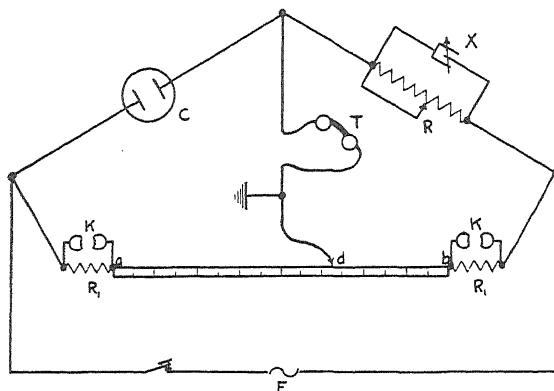


FIG. 1. Diagram of Wheatstone bridge for measurement of electrolytic resistance.

particularly valuable in stimulating accurate work in conductivity measurements. Of particular importance from the standpoint of precision measurements has been the introduction of vacuum tube alternating current generators and amplifiers for providing currents of symmetrical wave form, on the one hand, and increasing the sensitivity of the detector on the other (11, 19, 20). JONES and JOSEPHS (20) have described in a detailed paper a direct reading alternating current bridge embodying these new features. Their paper also includes a valuable and comprehensive study of various sources of error from the electrical standpoint in the Kohlrausch method as it has been generally used. STONE (35) has likewise described a vacuum tube potentiometer for the measurements of the conductivity of solutions. The circuit employs two thermionic tubes, the plate currents of which are visually balanced by a very sensitive null-point galvanometer through the adjustment of grid potentials derived from small batteries, and the superimposed alternating current used in the measurement.

For wiring diagrams of modifications and improvements over the classical Kohlrausch method the reader is referred to articles by HALL and ADAMS (11), SHEDLOVSKY (33), and STONE (35).

#### Factors affecting accuracy of conductivity measurements

In measuring the conductivity of solutions it is desirable to use a low voltage in the bridge. This may mean a modification of the oscillator to give low and controllable voltages. The use of low voltages in the bridge diminishes its sensitiveness, and, as a result, the amplification must be improved. The principles of design of a transformer for a vacuum tube amplifier are described by JONES and BOLLINGER (19) and STONE (35).

The resistances used must be as free as possible from the effects of inductance and capacitance at the frequency of the current in the bridge



circuit; also, attention must be given to protecting the bridge against external influence caused by capacitance and inductance. JONES and BOLLINGER (19) state that the mutual inductance between the oscillator and the detector, and between the oscillator and the bridge, may cause an error in the measurement of the conductivity of solutions when low voltages are being used. They give means for the discovery and elimination of this error.

For precision work, the electrostatic screening of the bridge is essential. An unscreened bridge is affected by the movements of the observer. Without adequate shielding, delicate balances are difficult to obtain because of shifting capacities introduced by the hand of the operator, etc. Also, only with proper screening are measurements of high precision possible under conditions existing in modern laboratories in which other electrical work may be in progress.

The experimenter must determine the desired accuracy needed in the oscillator, the current detector, etc. References on these points can be found in papers by SHEDLOVSKY (33) and WASHBURN and PARKER (41).

Balancing of the circuit depends upon instantaneous equality of potential at the terminals. This condition cannot be established if the currents in the two parts of the bridge are out of phase. If the conductivity of the electrolyte is high the current flowing in the cell may be out of phase with that of the resistance on account of the electrostatic capacitance of the conductivity cell. Such a condition prevents perfect balancing of the system. In precise measurements an adjustable condenser is connected across the terminals of the balancing resistance to annul the capacity of the cell.

The use of properly constructed conductivity cells and platinized electrodes is essential. The use of cells of high cell constant (length divided by cross section) is recommended so that the resistance to be measured will be high (10,000 to 30,000 ohms), thereby reducing polarization and heat effects in the cell. A convenient test of the quality of cells is observation of the variation in apparent resistance with variation in frequency. Cells with sliding electrodes, or electrodes easily distorted, should be avoided. The reader is referred to a paper by WASHBURN (38) for different types and sizes of conductivity cells adapted to measurement of solutions possessing low to high conductivity. He gives formulae to calculate the area of electrodes and the distances between these electrodes to be used for different ranges of conductivities, together with the minimum volume for each type of cell.

In most cases the platinum electrodes should be coated with platinum black, which increases the surface area and aids in reducing the surface density of ions deposited, to which the E. M. F. of polarization is proportional. The platinum electrodes must be thoroughly cleaned before being

coated with platinum black. This may be done by pouring enough concentrated HCl into the cell to cover the electrodes. The electrodes are connected with a current of 1.5 to 6 volts through a reversing switch and then electrolyzed by reversing the current every 30 seconds until all of the old platinum black is stripped off. The hydrochloric acid is then poured out and the cell rinsed. Now the electrodes are covered with platinizing solution and electrolyzed with 30-second reversals for 10 minutes or more. A resistance is often inserted in the system so that a gentle evolution of gas occurs, and a fine deep black coating of platinum, free from lead, is deposited. The platinizing solution consists of 1 gm. of chloroplatinic acid and 0.008 gm. of lead acetate in 30 ml. of water. After the electrodes are well coated with platinum black, the cell is immersed for a number of hours in warm distilled water, frequently changed to remove absorbed salts. Finally, 6 N  $\text{H}_2\text{SO}_4$  is poured into the cell which is connected with the source of the current and electrolyzed with 30-second reversals for a period of about 15 minutes. This treatment removes from the electrodes the last traces of platinizing solution and occluded chlorine. The electrodes are then rinsed and stored in distilled water. They should not be allowed to dry out.

Conductivity cells for low conductance solutions should be lightly platinized and those for high conductivity solutions should be heavily platinized. The cells should be carefully annealed and aged before use. Likewise, large temperature changes should be guarded against as they lead to thermal strains which may alter the distance between electrodes.

The cell constant applies only to a particular temperature. Kohlrausch has recommended several solutions of known conductivity for standardization of the electrolytic cell, but in many cases these are suitable only for approximate work. His standard is normal KCl solution made up from 74.555 gm. of pure KCl weighed in air and diluted to one liter at 18° C. This solution should have a conductivity of 0.09822 and a density of 1.04492 at 18°. Frequently the normal solution of KCl is diluted to 0.02 N and used for determining the cell constant. This solution at 18° has a conductivity of 0.002397 according to Kohlrausch.

PARKER and PARKER (29) recommend that, with the ordinary definition of cell constant, the concentration of the solution should be expressed in equivalents per cubic decimeter, not per liter, and unit concentration is then demal, not molal or normal. They also find that the value of the cell constant depends on the voltage and frequency of the current, and on the electrolyte used. They suggest that the standard solution be made from 76.6276 gm. of pure KCl weighed in air and added to 1000 gm. of water also weighed in air. The standard solution may then be diluted as in solutions recommended by Kohlrausch to 0.1 to 0.01 N. The conductivity of the

one demal solution was found by PARKER and PARKER (29) to be 0.097790 at 18°. This value is lower than that given by Kohlrausch. Tables of values for temperatures from 0° to 30° are given along with a comparison of previous data.

WASHBURN (38) determined the cell constant with a solution of 7.4300 gm. KCl in 1000 gm. of solution (weights in air). This solution produced a conductivity of 0.01288 at 25°, in contrast with that of PARKER and PARKER, of 0.0128524 at 25°. It is good practice to ascertain the known conductivity of a given solution as recorded in the International Critical Tables.<sup>2</sup> The writer's experience has shown that these tables as given in textbooks may contain errors.

It is necessary to have a balance between the resistance of the lead wires to the conductivity cell and that of the wire connecting the resistance box with the bridge. Otherwise the lead resistance must be determined by measuring the resistance of the cell when filled with mercury and this resistance subtracted from the measured resistances.

Alternating current of high frequency is necessary to prevent the polarization of the electrodes which occurs when direct current is used (36, 38). Currents of 1000 cycles or more should be used to minimize polarization. Polarization can be minimized and made practically insignificant by a combined use of proper platinization, large electrodes, high frequency, and high resistance. Experiments have shown that, if polarization is eliminated, Ohm's law can be accurately followed, and the actual values of the applied potential and current need not be known as they cancel out in the final calculation of conductivity.

Since the resistance of an electrolyte is affected appreciably by temperature, the conductivity cell must be immersed in a bath, the temperature of which is kept constant and measured accurately. Water may be used as a bath liquid, but in some cases in which the conductivity of the electrolyte is low, an oil bath is preferable. A temperature difference of 1° C. changes the value of the conductivity of the solution about 2 per cent. In order to obtain an accuracy to 0.1 per cent., temperature control of 0.05° is necessary.

Good insulation between the various parts of the apparatus is essential.

### Conductivity measurements in pure and biological solutions

The conductivity of pure liquids is small. Purest water obtained by KOHLRAUSCH and HEYDWEILLER (23) had a conductivity of  $0.043 \times 10^{-6}$  at

<sup>2</sup> JONES, G., and PRENDERGAST, M. J. Jour. Amer. Chem. Soc. 59: 731-736. 1937: "the absolute specific conductance of the 1, 0.1 and 0.01 N KCl solutions recommended by Kohlrausch as a standard of reference for conductivity measurements have been re-determined. The results indicate that the corrections used by the 'International Critical Tables' in the recalculation of the conductance data are unreliable."

18°. If pure water is allowed to reach equilibrium with the  $\text{CO}_2$  of the air, its conductivity increases and is about  $0.8$  to  $1 \times 10^{-6}$ .

The interpretation of conductivity values obtained from single component solutions, pure and dilute, is much simpler than those secured from mixed or biological solutions. The conductivity values obtained from plant saps<sup>3</sup> must be interpreted cautiously. It is almost impossible to translate conductivity values of mixed salts or plant saps into concentration values. Accordingly, one cannot calculate from electrical conductivity measurements the concentration of salts in plant saps. DEXTER (3) and others have attempted to translate conductivity values into salt concentrations. DEXTER states that "the salt per gram of water in the plant decreased as the season advanced, in spite of the fact that the amount of water decreased. That is, the concentration of salt in the sap, assuming all water to be free for solvent purpose, decreased regularly." HARRIS, GORTNER, and LAWRENCE (12) have suggested the use of the ratio  $\frac{k}{\Delta}$ , as of value in indicating changes in the ratio of electrolytes to non-electrolytes in plant saps. PASCOE (30) found that the depression of the freezing point in the mixture of  $\text{MgCl}_2$  and  $\text{MgSO}_4$  is a linear function of the mixture, whereas the conductivity curves is not a linear function in mixed solutions. PASCOE's results, as well as the writer's (7, 8), indicate that the conclusion of DEXTER and the use of a ratio by HARRIS, GORTNER, and LAWRENCE are unwarranted.

Factors that may affect the conductivity of plant saps are: (1) hydration of colloids and crystalloids (frequently recorded as "bound water" or "unfreezable water"); (2) viscosity; (3) adsorption of ions by colloids (colloids differ in their ability to adsorb certain ions (16)); (4) nonelectrolytes; (5) change in the dielectric constant of the system, as a result of the action of colloids, organic acids, etc.; (6) surface conductance of colloids; (7) concentration and solubility of the crystalloids; (8) temperature; and (9) size of molecule of crystalloids.

WIEDEMANN (42) was the first to point out a connection between conductivity and viscosity. From his work on solutions of  $\text{CuSO}_4$ , he formulated a relation  $\frac{ky}{p} = \text{constant}$ , where  $k$  is the conductivity of a solution of concentration  $p$ , and  $y$  is its viscosity. The resistance offered to the motion of an ion in solution because of the viscosity may be expected to have some influence on conductance. For single component systems of higher concentrations, the calculated ionization has been shown to change by 8 per cent. depending upon whether or not viscosity is taken into account. It is quite evident that viscosity influences the conductance of plant saps (tables I, III).

<sup>3</sup> For a discussion of methods of extracting of plant saps see *Plant Physiol.* 4: 103. 1929; 7: 439. 1932.

GREEN (10) working with sugar, salt, and acid solutions, showed that conductivity did not vary directly as fluidity, but to some function approximately  $f^{2/3}$ . The power 0.55 was found to give the best results for the experiments with HCl, and 0.70 when KCl was the electrolyte used.

Table I presents representative data that indicate the influence of some of the factors involved in the interpretation of conductivity measurements on plant tissues. The red clover plants used in this study were a portion of the samples collected on January 30, 1935, for which a chemical analysis has been published (8). The cabbage plants were of the Early Jersey Wakefield variety. These plants were grown until 67 days of age under identical cultural and environmental conditions. At the end of this period the plants were divided into two groups. One group of plants was left in the greenhouse while the other group was placed in the hardening room at 40° F., remaining there for an additional period of 12 days. The plants were illuminated by means of 100-watt Mazda lamps placed at a distance of 3 ft. from the plants. During the growth of these plants, an attempt was made to control all variables except the temperature at which the plants were grown for the 12-day period previous to sampling. The *Bryophyllum* plants were grown in the general plant physiology greenhouse. The methods used in these analyses were similar to those previously discussed (7, 8).

TABLE I  
PHYSICAL AND CHEMICAL MEASUREMENTS OF PLANT TISSUES

TISSUE	TOTAL MOIS- TURE	UN- FREEZ- ABLE WATER - 22° C. % TOTAL WATER	SPE- CIFIC CON- DUC- TANCE $\times 10^{-5}$ $25 \pm$ $0.02^\circ$ C.	VIS- COSITY (REL- ATIVE)	PH	TOTAL SUGAR % DRY WT.	PRO- TEIN NITRO- GEN* % DRY WT.	NON- PRO- TEIN NITRO- GEN* % DRY WT.
	%	%				%	%	%
Cold-hardened red clover roots								
French variety ...	80.60	17.37	1069	1.146	5.24	10.86	1.527	1.278
Ohio variety .....	77.90	20.46	865	1.250	5.57	13.40	1.526	1.326
Cabbage (shoot) 79 days old								
Unhardened .....	90.32	8.30	1637	1.356	5.26	5.68	1.617	0.532
Hardened .....	88.32	9.53	1331	1.407	5.32	8.23	1.376	0.766
<i>Bryophyllum</i>								
Leaf blade .....	93.79	1.59	734	1.136	4.03	.....	.....	.....
Young plants from leaf blade .....	83.25	4.18	616	1.251	4.49	.....	.....	.....
Leaves from three lower nodes .....	93.60	1.76	741	1.146	4.12	.....	.....	.....
Leaves from three upper nodes .....	91.73	3.25	625	1.198	4.83	.....	.....	.....

\* The nitrogen determinations were made by DR. NEIL W. STUART.

In the interpretation of the data of table I it is essential to keep in mind that the carbohydrate, nitrogen, total water, and unfreezable water analyses were made on the whole tissue, while the conductivity, viscosity, and pH determinations were made on the plant sap. The data of table I show that with each plant type the conductivity varied inversely with the relative viscosity, the unfreezable water, and the total sugar content. These data show that the total water content is positively correlated with the conductivity, while the unfreezable water and nonprotein nitrogen values are negatively correlated with conductivity. No consistent relationship between protein nitrogen and conductivity was found. It has been previously pointed out that there was no consistent relationship between total water content of roots and shoots and the hydration measurements (9). Neither was the protein nor the nonprotein nitrogen found to influence markedly the hydration capacity of the tissues.

KRUYT (24) states that investigations made in his laboratory have forced him to assume that hydration, which is without doubt closely connected with the orientation of the water dipoles, must be understood as meaning that the dipoles which are situated in the neighborhood of a particle are in complete orientation and that those somewhat more remote are less perfectly orientated. If this is the case, the degree of hydration would greatly influence the solubility of the salts present, and the tissue with the greater degree of hydration would have the lower conductivity.

It is difficult to distinguish cause from effect in conductivity measurements of plant sap. Nevertheless, with a critical examination and application of both physical and chemical methods of analysis to plant tissues the relation between cause and effect of conductivity of plant saps should become clearer.

The same basic factors may be responsible for lower conductivity in cold-hardened tissue and in tissue of different age and position on the same plant (table I). Other investigators, when studying the gradients in plants, have noted a lower conductivity with younger tissue. This shows how difficult it is to interpret conductivity measurements even within the same plant. From the data in tables I and II and from those of other investigators, evidence points to the possibility that ions bound to the colloidal phase could offer considerable protection to loss of water from plant tissues.

In order to determine whether this lower conductivity was due to lower ion or salt content, sap was expressed from plant tissues, the conductivity determined, and 50 ml. of this plant sap brought to volume in an accurate thermostat. This volume of plant sap was electrodialed until free of all removable ions and again brought back to the original volume and the conductivity redetermined. The results of this experiment are presented in table II.

TABLE II

CONDUCTIVITY MEASUREMENTS ON PLANT SAP BEFORE AND AFTER ELECTRODIALYSIS

PLANT SAP FROM	CONDUCTIVITY OF SAP BEFORE ELECTRO- DIALYSIS $\times 10^{-5}$ $25 \pm .02^\circ \text{C.}$	CONDUCTIVITY OF SAP AFTER ELECTRO- DIALYSIS $\times 10^{-5}$ $25 \pm .02^\circ \text{C.}$	DIFFERENCE
Cold-hardened red clover roots			
French variety .....	1069	1106	37
Ohio variety .....	865	1023	158
Cabbage plants 79 days old			
Unhardened .....	1637	1698	61
Hardened .....	1331	1601	270
Unhardened red clover roots			
French variety .....	873	895	22
Ohio variety .....	708	726	18

These data indicate that the differences in conductivity in these tissues are not caused entirely by potential ion content, but that some of the ions are probably adsorbed, or that ionization of the salts is influenced by viscosity, dielectric changes, etc.

The influence of non-electrolytes on conductivity of salt solutions has been known for many years. Very few, if any, measurements have been made of the influence of nonelectrolytes on plant saps. Table III gives data to show the effect of sucrose on the conductivity of plant saps.

TABLE III

EFFECT OF SUCROSE ON CONDUCTIVITY OF PLANT SAP

SUCROSE ADDED TO PLANT SAP	CONDUCTIVITY OF PLANT SAP $\times 10^{-5}$ $25 \pm .02^\circ \text{C.}$		DIFFERENCE
	ORIGINAL	AFTER ADDITION OF SUCROSE	
Normality			
0.02 .....	805	788	17
0.1 .....	805	745	60
0.2 .....	805	701	104
0.4 .....	805	724	81

It can be noted from table III that the conductivity of the plant sap decreases on the addition of sucrose in small amount, probably because of decrease in ionization and the mobility of ions as a result of change in viscosity. With further addition of sucrose the conductivity increased, possibly because of hydration and concentration effects on the nonelectrolytes. The addition of other sugars, such as glucose and fructose, to plant saps gave results similar to those of the experiments with sucrose differing, however, in the concentration needed to produce a decrease in conductivity. The results in table III were corrected for the conductivity of the sugar solutions.

The conductivity and the freezing point depression of solutions of sucrose in distilled water were determined in order to note the influence of different concentrations. These results are presented graphically in figure 2.

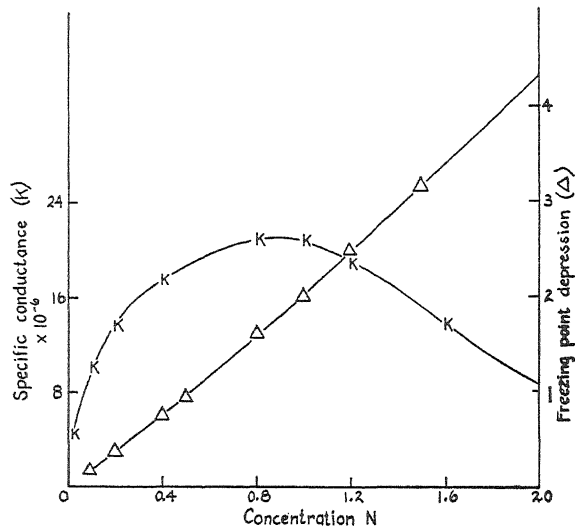


FIG. 2. Variations of specific conductance and freezing point depression with change in concentration of sucrose.

The conductivities shown by these sugar solutions are probably caused, not only by small quantities of impurities present, but also by a slight ionization of the sugar molecule itself, since other workers have shown that sucrose is to be regarded as an extremely weak acid. The decrease in conductivity after the solution has reached a concentration of 0.8 N is probably the result of a decrease of ionization and mobility of the ions.

The depression of the freezing point curve for sucrose deviates progressively from a straight line in a manner indicative of a greater apparent degree of hydration of the sucrose molecule at higher concentrations. In other words, the sucrose does not attract the  $(\text{HOH}_n)$  molecules to an extent always equivalent to the formation of a hexahydrate, as suggested by SCATCHARD (31), but increases in its apparent degree of hydration with decrease in the relative activity of the water in the solution. This may have some bearing on the conductivity values of plant saps.

As it was thought possible that inversion of the sucrose might be brought about by the presence of materials in the plant sap, with a consequent small alteration of the viscosity, a solution was kept for an hour and examined by polariscope and reduction, but little inversion could be detected, and the effect of such a source of error may be dismissed as negligible.

There are a number of other possible factors that might be responsible



for deviations in conductivities of plant saps. MARINESCO (26) has found that the dielectric constant is lowered by the addition of colloids to the solvent water.<sup>4</sup> On the basis of these findings it is probable that the dielectric constant is lowered by water-binding of the cell constituents. Therefore, the tissue with the greater water-binding capacity should have less dissociation of electrolytes and consequently a lower conductivity. A number of investigators have noted a change in organic acids with change of the environment of the plants. These changes in acid content would likely influence the hydration of the system and its dielectric value.

There is always the possibility that a certain portion of the conductivity in plant saps is caused by the surface conductance of colloids. BRIGGS (2) found that the electrical conductivity of a colloid gel was not necessarily related to the presence or the concentration of ionized electrolytes. He noted that a membrane of pure cellulose immersed in conductivity water conducted a current, and he was able to demonstrate that the conductivity was not due to inorganic constituents present in the system. In a more recent paper, BRIGGS has demonstrated that there is a definite lyotropic series of ions which influence surface conductance. As a result of these investigations there is indication that the conductivity of plant saps is in part associated with surface conductance through the lyophilic colloidal gel structure, as well as with ionic conductance of the solvent.

### Application of conductivity measurements

Pure water is essential for most laboratory work and it is necessary to know from time to time the conductivity of the distilled water used as a solvent. These data can be obtained quickly and accurately by means of the conductivity apparatus. Distilled water for general laboratory purposes should not have a conductivity greater than from  $1.5$  to  $2 \times 10^{-6}$  and for work of greater precision water of conductivity  $1 \times 10^{-6}$  or better should be obtained. In precision conductivity measurements there is a water correction. This correction has been discussed by WASHBURN (39) and by KENDALL (21, 22).

The principle of increased permeability and electrical conductivity of injured tissue is a familiar one to the plant physiologist. The application of this method to different plant problems can be found in papers by HEALD (14), HIBBARD and MILLER (15), HOAGLAND and DAVIS (17), HOTTES (18), MERRILL (27), and OSTERHOUT (28).

The diffusion of electrolytes from tissue exposed to low temperature in distilled water has been determined electrometrically by DEXTER and asso-

<sup>4</sup> In this connection it is interesting to note that ALEXANDER and SHAW (1) have recently devised a method for the determination of ice-water relationships by measurements of dielectric constant changes.

ciates (4). The magnitude of the electrical conductivity has been associated with the known hardness of the tissue. GREATHOUSE and STUART (7, 8) have applied conductivity measurements of plant sap to the problem of cold hardness. According to the work of DEXTER, and of GREATHOUSE and STUART, the degree of cold hardness of plants is related to the ions that move by diffusion from the tissue or to the ions free in the plant sap. This measurement, however, does not give the true ionic relationship in these tissues (tables II, III).

The production of  $\text{CO}_2$  in respiration has been measured electrometrically (13, 34).

Conductivity measurements are very useful in determining when the substrata are free of their ions after electro dialysis of plant materials.

The physical chemist has used this measurement for the determination of (a) the solubility of difficultly soluble salts; (b) the basicity of organic acids; (c) the degree of hydrolysis; (d) the end-points in titration reactions; and (e) the dissociation of water and other solutions.

### Precautions in electrical conductivity measurements

If the proper apparatus is assembled, connected, and adjusted correctly it is possible to make conductivity measurements with the great degree of precision which is necessary in all research laboratories in plant physiology. The application of this measurement to some of our plant problems has proved of great importance. The method has practical value even though its results cannot be interpreted in terms of ion concentration and behavior. If the true ion concentration and behavior are required, it will be necessary to determine these properties through additional experimentation.

It is obvious that anyone who is to make conductivity measurements should know the basic principles of the apparatus as well as those of the ionic relationships of solutions. One should know how to design and platinize electrolytic cells for solutions of low or high conductivities. Other factors of equal importance have been previously mentioned in this paper.

Errors in the conductivity measurements must be guarded against if it is to be a useful tool to plant science. Errors produced by polarization at the electrodes, changes in temperature, etc., on conductance are or should be common knowledge. It is the less obvious errors that are frequently ignored.

### Electrical units and laws pertaining to conductivity

Ohm, in the well known law ( $E = RIk$ ), summarized the relation between resistance, current, and pressure, where  $R$  is the resistance,  $E$  the electric pressure, and  $I$  the current, while  $k$  is a factor depending only on the choice of units and can be made equal to unity.

The unit of electrical resistance is the International Ohm and is defined as the resistance offered to an unvarying current by a column of mercury at the temperature of 0° C., 14.4521 gm. in mass, 106.3 cm. in length, and of constant cross section.

The unit of current is the International Ampere and is defined as the unvarying electric current which when passed through a solution of silver nitrate in water, in accordance with specified conditions, deposits silver at the rate of 0.001118 gm. per second.

The unit of electric pressure is the International Volt and is defined by Ohm's law as the electric pressure which, when steadily applied to a conductor the resistance of which is one International Ohm, will produce a current of one International Ampere.

Both the ampere and the volt are based upon the fundamental law of Faraday, which shows the relation between the current and the amount of chemical reaction that takes place when a current passes from a metallic conductor to an electrolytic conductor. Faraday's law is divided into two parts and may be expressed by the following relationships: (1) the amount of any substance deposited by the current is proportional to the quantity of electricity flowing through the electrolyte, (2) the amounts of different substances deposited by the same quantity of electricity are proportional to their chemical equivalent weights.

The specific resistance, or resistivity, of an electrolyte may be defined as the resistance in ohms of a centimeter cube of solution. Specific conductance, or conductivity, is the reciprocal of specific resistance.

The molar conductance is the conductivity, in reciprocal ohms, of a solution containing one mol of solute when placed between sufficiently large electrodes which are exactly 1 cm. apart. The equivalent conductance may be defined in the same terms as molar conductance, if one gram-equivalent of solute is substituted for one mol of solute.

It is obvious that the construction of an electrolytic cell with electrodes suspended exactly 1 cm. apart and each electrode having 1 sq. cm. of area, would be difficult. To avoid this difficulty a correction is made and is incorporated in the value known as the cell constant.

The cell constant,  $C$ , is defined by the equation  $k = C/R$  in which  $k$  is the specific conductance of a solution and  $R$  the resistance of the cell containing that solution. The cell constant may be calculated from a measurement of  $R$  by the usual procedure when a solution (usually 0.1 or 0.01 N KCl) of known  $k$  is in the cell.

### Summary and conclusions

1. The basic principles, the apparatus, and the precautions needed in determining the specific conductance of solutions are discussed.

2. Data are presented which indicate that conductivity values obtained from plant sap must be interpreted cautiously.

3. A list of possible factors that may affect the conductivity values of plant sap is presented and each factor discussed.

4. The electrodialysis data indicate that the lower conductivity of the expressed sap from cold-hardened plants was not due entirely to fewer ions, but was probably influenced by adsorption, viscosity, ionization, etc.

5. The conductivity of plant sap was found to decrease on the addition of nonelectrolytes in small amounts. With further addition of nonelectrolytes the conductivity increased.

6. The interaction of the solute and solvent is of major importance in the interpretation of electrical conductivity data.

7. The application and limitations of specific conductance measurements are given.

BUREAU OF PLANT INDUSTRY  
COLLEGE STATION, TEXAS

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# BEHAVIOR OF PECTIC SUBSTANCES AND NARINGIN IN GRAPEFRUIT IN THE FIELD AND IN STORAGE

G. L. RYGG AND E. M. HARVEY

(WITH FIVE FIGURES)

## Introduction

The physiological breakdown of citrus fruits variously known as storage spot, pitting, or pox has presented a perplexing problem to growers and shippers ever since the storage and transportation of these fruits was undertaken on an extensive scale. This breakdown manifests itself in various forms of blemishes of the rind, depending on such factors as the inherent condition of the fruit, the temperature at which the fruit is held, the length of storage, and the composition of the atmosphere surrounding the fruit. The disease is associated with exposure to excessively low temperatures, but its development is conditioned by the other factors mentioned. Partial control of this disorder has been attained in commerce by raising the temperature at which the fruit is held during transit and storage, the temperature depending on the kind of fruit in question. California oranges hold well at 38° F., but California-grown lemons and grapefruit require a temperature of 50° to 55°, and recommendations of temperatures as high as 65° have been made for commercial grapefruit storage. Other factors enter into the consideration of the best holding temperature; *e.g.*, grapefruit will develop a deeper yellow or bronze at a temperature of 55° than at 65°. This deep yellow color is considered undesirable by the trade because of the appearance but has no ill effect on the quality of the fruit. On the other hand, fruit held at 65° will be more subject to attacks by the various organisms of decay. High relative humidities are necessary at these higher temperatures in order to reduce shrinkage, but also afford favorable conditions for the development of these organisms.

It has been shown previously (6) that losses from pitting in grapefruit can be reduced by choosing the proper season for picking fruit which is to be held or which is to be shipped great distances under refrigeration. Additional evidence on the influence of season on the susceptibility of grapefruit to pitting will be submitted in this paper. The nature of the changes occurring in the fruit during its development on the tree which tend to increase or decrease its susceptibility to pitting if subjected to the unfavorable environment of low storage temperatures is not known. Evidence has been presented previously (6) and additional evidence is presented in this paper indicating that the temperature in the grove during the period immediately preceding picking has a bearing on the susceptibility the fruit will have to

pitting upon subsequent storage. Evidence is also presented that certain concomitant changes take place in the composition of the rind of the fruit. These changes may have a partial responsibility in bringing about variations in susceptibility to pitting, but other changes which have not been noted may play a more important part.

### Material and methods

Although the general problem of the pitting of the rind arises in several kinds of citrus fruits, this report will be limited to work on the Marsh variety of grapefruit (*Citrus grandis* (L.) Osbeck). In order to obtain information on the possible effect of the place of production on the holding quality of the fruit, collections were made from three widely separated groves: (1) Near Oasis, California, in the Coachella Valley; (2) Corona, in the western part of southern California; and (3) Woodlake, in the central California citrus district. At Oasis the collecting period extended from October 29, 1935, to April 14, 1936; at Corona from March 10, 1936, to October 6, 1936; at Woodlake from December 4, 1935, to July 21, 1936. Each interval between collections was approximately 6 weeks. Five collections were made at Oasis and six each at Corona and Woodlake. At Oasis the collecting extended over a period of 168 days, at Corona 210 days, and at Woodlake 211 days. At each collection sufficient fruit was collected to provide 50 fruits for each storage room and 50 for immediate sampling. The fruit was collected from the same group of trees at each picking. Of each lot of 50 fruits 20 were used for the analytical work and the remainder for the respiration studies. The analytical material consisted of the flavedo and albedo from the stem end of the fruit. These lots were ground in a food chopper with a nut-butter attachment, mixed thoroughly, and samples weighed out for pectin, naringin, and water determinations. Duplicate 25-gm. samples were used for the pectins, duplicate 10-gm. samples for naringin and for the water content of the flavedo, and single 50-gm. samples for the water content of the albedo.

Pectic substances were determined as calcium pectate by the method used in work previously reported (6). Naringin was determined by the colorimetric method described by HARVEY and RYGG (5). Water content of the flavedo was determined by the toluene method and of the albedo by drying to constant weight at 80° C. The latter method was unsatisfactory for the flavedo because of the error introduced by the volatile oils.

The method used in the respiratory studies was that described by HARVEY and RYGG (7). In this method the fruit is placed in an airtight chamber fitted with a mercury manometer and the changes in pressure are recorded at frequent intervals. A blank with approximately the same free air space is observed simultaneously in order to provide corrections for barometric pressure changes and for changes in gas volume due to the slight temperature

variations. This correction would not compensate for any changes in rate of respiration brought about by temperature changes.

At the time the fruit is placed in these chambers it is surrounded by atmosphere of normal composition, but as the oxygen is consumed and  $\text{CO}_2$  is given off there is a gradual change to anaerobic conditions. During this change the pressure is being reduced.<sup>1</sup> The maximum negative pressure occurs at approximately the time when the  $\text{O}_2$  becomes exhausted; then with continued  $\text{CO}_2$  production the pressure increases at a fairly constant rate for a considerable period of time. The rate at which these changes in pressure occur at a given temperature depends on the condition of the fruit. It was found that with grapefruit there was a correlation between the length of time during which the pressure remained negative and the pitting which developed after subsequent storage of corresponding lots.

In all except rare instances respiration in stored fruit produced a negative pressure of varying intensity and duration followed by the development of positive pressure at a uniform rate for a period of days. The data presented are based on the duration of this negative pressure.

The storage rooms were maintained at 38°, 46°, 56°, and 68° F. Fruit from Oasis was not stored at 68°. No pectic analyses were made on stored Woodlake fruit nor on the stored fruit of the second picking from Corona.

## Results and discussion

### BEHAVIOR OF FRUIT IN STORAGE

The relation between the stored and field lots is shown in figure 1, and the severity of the pitting in tables I and II. Pitting was most severe in the

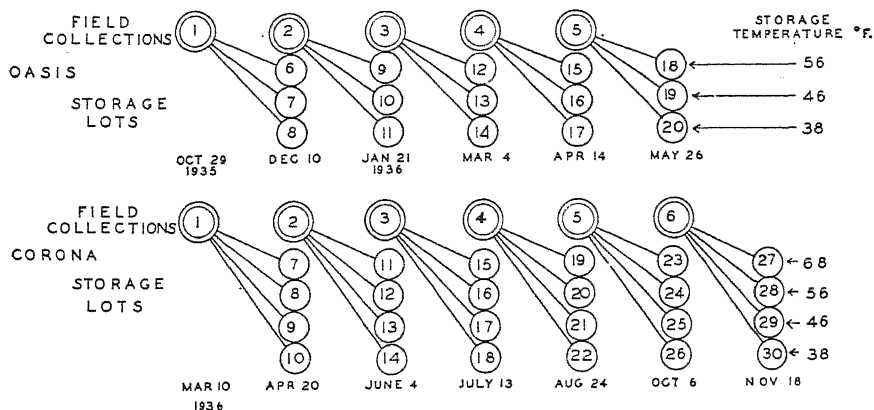


FIG. 1. Diagram showing relation of storage lots to various field conditions.

<sup>1</sup> This apparent reduction in gas volume is accounted for, at least in a large part, by the evolved  $\text{CO}_2$  going into solution in the fruit juice. This fact magnifies the need for a standard and constant temperature in using this method.

TABLE I

SEASONAL VARIATIONS IN SUSCEPTIBILITY OF MARSH GRAPEFRUIT TO PITTING AND IN COMPOSITION OF THE RIND WITH REFERENCE TO WATER, NARINGIN, AND PECTIC SUBSTANCES. OASIS, CALIFORNIA, 1935-36\*

DATE OF PICKING	LOT NO.	STORAGE TEMPERATURE	MOISTURE CONTENT		PECTIC SUBSTANCES						NARINGIN	PITTING		
			FLAVEDO	ALBEDO	FLAVEDO			ALBEDO				SLIGHT	SEVERE	TOTAL
					SOLUBLE	PROTEIN	TOTAL	SOLUBLE	PROTEIN	TOTAL				
		°F.	%	%	gm.	gm.	gm.	gm.	gm.	gm.	%	%	%	%
1935 Oct. 29 .....	{ 1 6 7 8 }	.....	74.3	72.3	1.5	12.7	14.2	2.5	13.9	16.4	.....	.....	.....	.....
		56	74.1	74.1	1.6	13.7	15.3	2.9	17.8	20.7	.....	0	.....	27
		46	72.0	74.1	1.0	12.0	13.0	2.3	17.2	19.5	.....	34	.....	70
		38	72.3	73.5	1.0	13.7	14.7	2.3	16.5	18.8	.....	66	.....	80
..... Dec. 10 .....	{ 2 9 10 11 }	.....	75.0	75.5	1.6	12.6	14.2	3.1	12.6	15.7	.....	.....	.....	.....
		56	77.9	75.7	1.8	14.3	16.1	2.7	14.0	16.7	.....	0	.....	2
		46	74.2	76.3	1.0	11.5	12.5	2.8	14.8	17.6	.....	12	.....	16
		38	74.7	76.0	1.3	13.6	14.9	3.7	13.3	17.0	.....	42	.....	76
1936 Jan. 21 .....	{ 3 12 13 14 }	.....	75.6	75.0	1.9	11.3	13.2	3.5	11.0	14.5	.....	.....	.....	.....
		56	77.9	78.2	1.1	11.3	12.4	3.9	14.2	18.1	.....	.....	.....	.....
		46	77.6	77.7	1.2	12.6	13.8	3.7	14.1	17.8	.....	66	.....	66
		38	77.6	77.8	1.3	11.2	12.5	4.4	12.4	16.8	.....	22	.....	58
..... Mar. 4 .....	{ 4 15 16 17 }	.....	78.8	81.3	1.7	12.5	14.2	5.7	11.3	17.0	.....	.....	.....	.....
		56	80.5	80.2	2.0	12.9	14.9	5.2	12.7	17.9	.....	0	.....	16
		46	79.1	81.2	1.7	12.5	14.2	5.4	10.7	16.1	.....	6	.....	78
		38	80.7	80.9	2.0	13.5	15.5	6.3	9.5	15.8	.....	96	.....	100
..... Apr. 14 .....	{ 5 18 19 20 }	.....	81.0	80.6	1.7	11.9	13.6	6.1	11.6	17.7	.....	.....	.....	.....
		56	79.1	80.7	1.1	11.4	12.5	5.3	12.7	18.0	.....	0	.....	8
		46	78.0	80.3	1.7	12.4	14.1	3.2	14.8	18.0	.....	40	.....	80
		38	77.7	80.6	1.5	14.0	15.5	4.5	13.4	17.9	.....	86	.....	100

\* Moisture is expressed in percentage of fresh weight, naringin in percentage of dry weight, and pectic substances in grams of calcium pectate per 100 gm. of dry tissue.

TABLE II

EASONAL VARIATIONS IN SUSCEPTIBILITY OF MARSH GRAPEFRUIT TO PITTING AND IN COMPOSITION OF THE RIND WITH REFERENCE TO WATER, NARINGIN, AND PECTIC SUBSTANCES. CORONA, CALIFORNIA, 1935-36\*

DATE OF PICKING	LOT No.	STORAGE TEMPERATURE	MOISTURE CONTENT		PECTIC SUBSTANCES				NARINGIN		PITTING	
			FLAVEDO	ALBEDO	FLAVEDO		ALBEDO		ALBEDO	%	SLIGHT	SEVERE
					SOLUBLE	PROTO-PECTIN	TOTAL	SOLUBLE	PROTO-PECTIN	TOTAL		
		°F.	%	%	gm.	gm.	gm.	gm.	gm.	gm.	%	%
March 10	1	68	77.0	78.2	1.4	10.6	12.0	6.0	9.8	15.8	8.90	0
	7	68	79.4	79.3	1.8	13.2	14.3	7.3	11.1	18.4	11.78	0
	8	56	78.3	78.2	1.6	13.2	14.8	6.6	11.0	17.6	11.69	0
	9	46	78.9	79.2	1.5	12.6	14.1	6.3	11.3	17.5	11.53	8
April 20	10	38	79.7	80.0	1.5	12.4	13.9	6.8	10.1	16.9	11.90	66
	2	68	77.3	80.6	1.4	9.9	11.3	5.6	10.4	16.0	10.10	0
	11	68	79.8	80.5	.....	.....	.....	.....	.....	.....	12.20	0
	12	56	79.8	80.0	.....	.....	.....	.....	.....	.....	11.48	0
May 4	13	46	.....	80.0	.....	.....	.....	.....	.....	.....	10.50	10
	14	38	.....	80.1	.....	.....	.....	.....	.....	.....	10.65	62
	3	68	79.4	80.1	1.5	10.5	12.0	5.9	8.6	14.5	9.90	0
	15	68	81.6	81.8	2.6	13.1	15.7	8.1	9.9	18.0	12.25	0
July 13	16	56	77.5	81.4	1.8	10.2	12.0	6.7	10.6	17.3	12.63	6
	17	46	80.4	79.4	1.8	13.3	15.1	6.9	9.3	16.2	10.09	12
	18	38	81.0	82.4	1.6	14.7	16.3	6.2	10.7	16.9	12.15	32
	4	68	82.0	81.0	1.6	11.6	13.2	6.3	10.7	17.0	11.15	0
August 24	19	68	81.5	81.0	2.3	14.8	17.1	6.8	11.3	18.1	12.21	0
	20	56	79.7	80.8	2.0	12.8	14.8	6.1	11.0	17.1	11.51	0
	21	46	80.4	82.1	2.1	14.1	16.1	3.8	12.8	16.6	12.12	12
	22	38	81.2	80.7	1.8	15.4	17.2	4.8	11.7	16.5	11.76	76
September 6	5	68	79.8	81.1	1.3	12.8	14.1	5.7	11.7	17.4	10.63	0
	23	68	81.8	80.8	2.9	14.5	17.4	6.3	12.6	18.9	12.76	0
	24	56	77.0	79.6	2.2	10.5	12.7	6.4	13.4	19.8	12.79	0
	25	46	79.8	80.3	1.4	12.9	14.3	3.9	13.9	17.8	12.33	4
October 6	26	38	79.8	80.4	1.6	14.3	15.9	5.3	11.4	16.7	11.42	54
	6	68	81.7	80.7	2.5	14.3	16.8	7.4	11.6	19.0	11.96	0
	27	68	81.5	82.3	1.3	11.9	13.2	7.8	10.2	18.0	11.41	2
	28	56	81.3	80.4	2.0	11.8	13.8	5.7	12.6	18.3	11.17	0
Total	29	46	82.1	81.1	2.0	12.6	14.6	5.1	13.4	18.5	11.85	82
	30	38	76.6	76.8	1.1	9.1	10.2	6.8	13.9	20.6	9.27	56

\* Moisture is expressed in percentage of fresh weight, naringin in percentage of dry weight, and pectic substances in grams of calcium pectate per 100 gm. of dry tissue.

fruit stored at 38° F. regardless of the source. Figure 2 shows the results obtained with fruit from Oasis, and figure 3 the results with fruit from

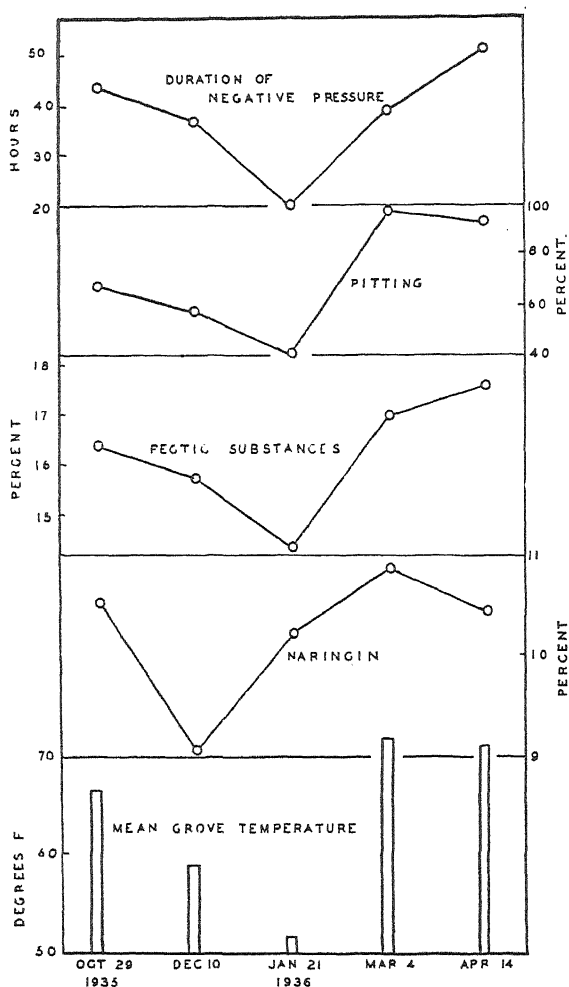


FIG. 2. Hours of negative pressure, weighted pitting percentage, total pectic content, and percentage of naringin of the albedo of the stem end of Marsh grapefruit grown near Oasis, California, with mean temperatures for the 5 days preceding picking.

Corona. The values used to express the severity of pitting were obtained by adding the percentage of severely pitted and one-half the percentage of slightly pitted fruit. This evaluation is arbitrary but was adopted for convenience. The grading of the pitting is also arbitrary; it is the same as that used in the previous report (6), namely, that pitting which was considered of commercial importance was classified as severe and anything less as slight.

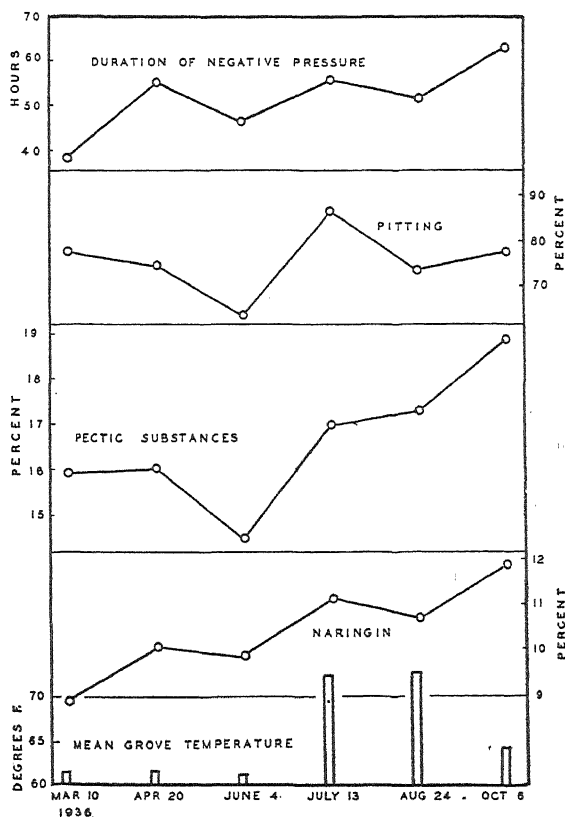


FIG. 3. Hours of negative pressure, weighted pitting percentage, total pectic content, and percentage of naringin of the albedo of the stem end of Marsh grapefruit grown near Corona, California, with mean temperatures for the 5 days preceding picking.

The fruit from Oasis pitted most severely early and late in the season with a period of reduced susceptibility in midseason. The susceptibility to pitting varied with the temperature prevailing previous to picking, being higher with the higher temperatures as shown in figure 2. The temperatures given are the averages of the mean daily temperature during the 5 days preceding the picking. Similar results were obtained with grapefruit from Oasis in 1933-34 and 1934-35, and from Phoenix, Arizona, during the season of 1934-35.

The fruit from Corona pitted with varying degrees of intensity during the season, but the least pitting also occurred about midseason at this location. There was considerable agreement between the mean temperature of the 5 days preceding picking and the susceptibility to subsequent pitting.

The fruit from Woodlake was highly resistant to pitting early in the season and became progressively more susceptible as the season advanced

until the last two pickings, when every fruit pitted and nearly all pitted severely. The first picking showed only 18 per cent. of slight pitting and no severe pitting after 6 weeks at 38° F. These results are in marked contrast with those obtained on fruit from Oasis which, if picked early, showed a high degree of susceptibility to pitting when stored at 38°. The relationship of pitting to the temperatures preceding picking was not so marked as with the Oasis fruit, although the lowest temperatures occurred previous to the first three pickings when the fruit was least susceptible to pitting.

#### RESPIRATION

It was found that the length of time during which the pressure remained negative differed considerably with fruit from the different groves; *e.g.*, this period of negative pressure developed by unstored fruit varied from 20 to 44 hours with fruit from Oasis, 40 to 68 hours with fruit from Corona, and from 17 to 82 hours with that from Woodlake. While the relationship of time of negative pressure to susceptibility to subsequent pitting holds roughly for fruit from an individual grove or group of trees, it does not hold when the behavior of fruit from one location is compared with that from another, perhaps many miles distant.

Fruit which had been stored and subsequently removed and placed in the chambers showed a speeding up of the metabolic processes which brought about these changes in pressure. This acceleration was proportional to the lowering of the storage temperature and the length of time during which the fruit had been held at these temperatures. It was also found that the rate as measured by the duration of the negative pressure was more rapid

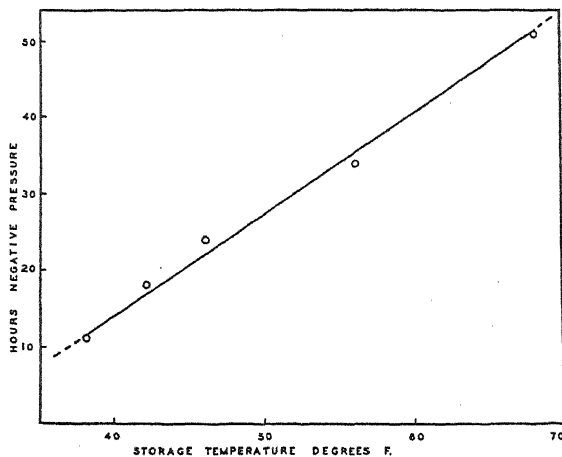


FIG. 4. The duration of the period of negative pressure developed by grapefruit in airtight chambers at room temperature subsequent to 6-weeks' storage at the temperature indicated.



in fruit which had pitted severely than in fruit from the same lots which had been held at the same temperature for the same period of time but which had pitted less severely or not at all. Figure 4 shows the relationship between the storage temperature and the hours of negative pressure developed by the fruit at room temperature. Each point on the graph represents the average of from 15 to 50 chambers.

The effect of the length of storage on the rate of subsequent anaerobic respiration at room temperature as measured by the duration of the negative pressure is shown in figure 5.

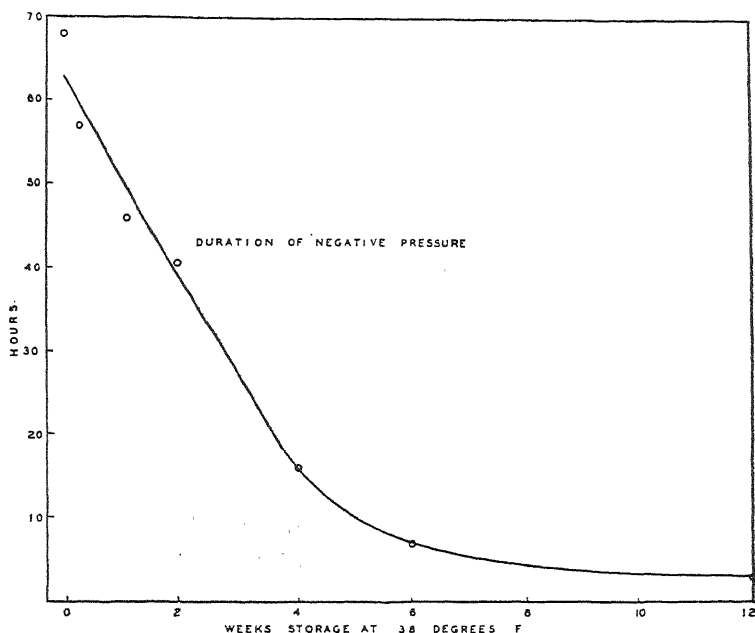


Fig. 5. The effect of length of storage period on the duration of the negative pressure developed. Storage at 38° F.

The fruit was placed in storage at 38° F. immediately after picking, except for one portion which was taken to the laboratory for immediate testing. The remainder of the fruit was removed from storage after intervals as indicated in figure 5, brought to the laboratory, and placed in the chambers. The duration of the period of negative pressure began to be reduced immediately after the fruit was stored. After 2 days' storage the period was reduced from 68 hours to 57 hours; additional tests were made after 8 days, and 2, 4, 6, and 12 weeks. The duration of the period of negative pressure varied inversely with the length of the storage period until pitting set in and the period of negative pressure had been reduced to a relatively

short time. After 4 weeks in storage the fruit had developed pitting to the extent of 18 per cent. severely pitted and 76 per cent. slightly pitted. All the fruit was severely pitted after 6 weeks in storage.

#### PECTIC SUBSTANCES

CHANGES IN PECTIC CONTENTS IN THE FIELD DURING THE HARVEST SEASON.—*Soluble pectin*.—The soluble pectin in the flavedo of the fruit from Oasis showed little change during the season. At the beginning of the season (October 29) there were 1.5 gm. of Ca pectate per 100 gm. of dry tissue; this increased to 1.9 gm. in midseason (January 21) and fell to 1.7 gm. at the end of the season (April 14). Soluble pectin in the albedo of the same fruit increased gradually throughout the season from 2.5 to 6.1 gm. This increase agrees with results obtained by GADDUM (4) on albedo of citrus fruit grown in Florida.

Soluble pectin in the flavedo of grapefruit grown at Corona increased from 1.4 gm. per 100 gm. of dry tissue on March 10 to 2.7 gm. on October 6. In the albedo the soluble pectin fluctuated somewhat during the season; the increase was from 6.0 to 7.4 gm.

In the flavedo of fruit from Woodlake the soluble pectin increased from 1.1 gm. on December 4 to 2.4 gm. on May 20, and dropped to 2.0 gm. by July 2. In the albedo of this fruit the soluble pectin increased throughout the season from 2.1 gm. on December 4 to 5.2 gm. on July 2.

In all instances the trend was for soluble pectin to increase during the season, though sometimes the increase was slight, especially in the flavedo. The albedo contained far more soluble pectin than the flavedo, the average of all field samples from Oasis being 4.2 and 1.7 gm.; from Corona 6.0 and 1.5 gm.; and from Woodlake 3.7 and 1.6 gm., respectively. There was no relationship between the soluble pectin content and the susceptibility of the grapefruit to pitting at any temperature.

*Protopectin*.—The protopectin content of the flavedo of fruit from Oasis varied somewhat, with the minimum occurring at the time of the third collection and the maxima at the beginning and the end of the picking season. In the albedo the minimum was also in the third collection and the maximum was in the first.

The protopectin in the flavedo of the fruit from Corona increased as the season progressed, with the minimum at the time of the second picking and the maximum at the end of the picking season. In the albedo the minimum occurred at the time of the third picking.

The protopectin in the flavedo of the fruit from Woodlake decreased throughout the season, falling from 13.8 gm. at the time of the first picking to 9.0 gm. at the end of the season. There was more fluctuation in the protopectin of the albedo, but there was no seasonal trend.

There was no trend in the changes in protopectin, which was true for both parts of the rind and for fruit collected from all locations. There was, however, a tendency for the minimum to occur at about midseason, especially in the albedo. This variability is not surprising in view of the variations in the climatic environment of the fruit from the different districts. It has already been shown that the climatic environment of citrus during its growth and immediately preceding its harvest has marked effects on the response of the fruit to treatments given after harvest. There appears to be a relationship between the protopectin of the rind, especially of the albedo, at the time of picking and the subsequent behavior of the fruit in storage, but the relationship between the total pectic content and the storage response is more apparent and will be discussed under that heading. The protopectin of the flavedo and albedo were practically equal. The average values for all the field collections were: Oasis, flavedo 12.2 gm., albedo 12.1 gm.; Corona, flavedo 12 gm., albedo 10.5 gm.; Woodlake, flavedo 11.6 gm., albedo 11.4 gm.

*Total pectic substances.*—The amount of total pectic substances in the flavedo of grapefruit from Oasis was lowest during midseason but the variation during the season was slight, ranging from 13.2 to 14.2 gm. There were considerably less total pectic substances present in the albedo during midseason than either before or after, the range in this tissue being from 14.4 to 17.7 gm. Figure 2 shows the amount at each picking and the relation to the mean temperature for the last 5 days before picking, respirational behavior, naringin content, and susceptibility to pitting.

The amount of total pectic substances of the flavedo of grapefruit from Corona increased from the second to the last collection, with the contents varying from 11.3 to 16.8 gm. There was more fluctuation in the total pectic substances in the albedo, where the minimum occurred in midseason and the maximum at the end of the season. The amount of total pectic substances in this tissue ranged from 14.5 to 18.9 gm. These fluctuations were apparently in response to environmental changes, as they coincided with the response of the fruit to storage conditions and to the respiratory behavior in tight chambers. These results are shown in figure 3.

The total pectic substances in the flavedo of fruit from Woodlake decreased during the season from 14.9 to 11.0 gm. In the albedo, however, the first two pickings gave the lowest yields of pectic substances and the last four the highest. The values ranged from 12.3 to 16.6 gm. The total pectic substances in the grapefruit from Woodlake did not correspond so well with the subsequent behavior of the fruit as did that from Oasis and Corona but it did agree in that in the albedo the lowest total pectic content and the least susceptibility to storage pitting occurred simultaneously, as did the highest pectic content and greatest susceptibility to pitting.

The nature of the relationship between the total pectic content of the

albedo and susceptibility to storage pitting is not known. At any rate, the response of the fruit on the trees to the environmental conditions were such that conditions which brought about a reduction in the total pectic substances in the albedo also brought about such changes as would reduce the susceptibility of the fruit to pitting at low temperatures. These results indicate that the condition and composition of the albedo at the time of picking are important in determining whether or not the fruit is likely to develop pitting if held at low temperatures.

The amount of total pectic substances in the flavedo held a similar relationship to subsequent pitting but the yield was less variable, hence the differences failed to show so distinctly. The flavedo of the fruit from Woodlake did not give results agreeing with the others as the pectic content decreased during the entire season.

The pitting resulting after 6 weeks storage at 46° F. also agreed with the results mentioned above, but the severity of the pitting was reduced at this temperature.

A comparison of the total pectic contents of the flavedo and albedo shows the albedo to be consistently but not greatly higher. Average values for all field collections from the various districts are: Oasis, flavedo 13.8 gm., albedo 16.3 gm.; Corona, flavedo 13.7 gm., albedo 16.7 gm.; Woodlake, flavedo 13.2 gm., albedo 15.1 gm.

CHANGES IN THE PECTIC CONTENTS OF STORED FRUIT.—The relationship in the proportion of soluble pectin to protopectin did not change materially in grapefruit stored 6 weeks at the temperatures mentioned. A surprising result, however, was the frequent apparent increase in total pectins at the end of these storage periods. Table III shows the average pectic content of the flavedo and albedo for a total of ten storage lots for each temperature

TABLE III

TOTAL PECTIC SUBSTANCES IN THE RIND OF THE STEM END OF MARSH GRAPEFRUIT BEFORE STORAGE AND AFTER STORAGE FOR 6 WEEKS, EXPRESSED AS GRAMS  
CA PECTATE PER 100 GM. DRY TISSUE

	NO STORAGE	STORAGE			
		68° F.*	56° F.	46° F.	38° F.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
FLAVEDO .....	13.7	15.5	13.9	14.2	14.7
ALBEDO .....	16.5	18.3	18.1	17.6	17.4

\* Figures in this column are for the fruit from Corona only, and represent the average of 5 pickings; all other values are for the fruit from Corona and Oasis, and represent a total of 10 pickings.

given and a corresponding number of fruit sampled immediately after collection.

In every instance the value for the stored lot was higher than that for the corresponding unstored lot. Also, there was a tendency for the value to be highest for the fruit stored at the highest temperature. BUSTON (3) indicates that high water content favors pectin production in growing tissue. No such condition is indicated in this instance, as the water content of these lots of fruit show very little variation, being 78.5 per cent. for both the flavedo and albedo of the field collections and 78.4, 78.3, and 77.7 per cent. in the flavedo and 78.9, 79.2, and 79.0 per cent. in the albedo of the fruit stored at the temperatures of 56°, 46°, and 38° F., respectively. Furthermore, this was not growing tissue. According to ONSLOW (8), however, the production of protopectin is brought about in steps by the inversion and oxidation of glucose and the entire system is in a state of flux. The reactions within the system are probably controlled by the "level of oxidative activity" of the cells (1). Consequently the direction and magnitude of the reactions are dependent on the factors that control this level. The apparent increase in total pectic content was brought about principally by increases in the protopectin fraction; out of 70 samplings there was an increase in soluble pectin in 41 instances, or 59 per cent. of the lots. In the same number of lots protopectin increased in 54 instances, or 77 per cent. of the lots, and total pectic substances increased in 73 per cent. of the lots. All previous reports of pectic changes of stored fruit have shown decreases. No doubt this would have occurred in grapefruit provided the fruit had been held long enough. Figures presented by BRANFOOT (Carré) (2) show that in apples the fruit was stored 17 weeks before the total pectic content began to decrease. No such long storage periods have been attempted in the present studies on pectic substances in grapefruit. BRANFOOT (2), however, found that pectose began to decrease and the soluble pectin to increase immediately after the fruit had been placed in storage, but this behavior has not been noted for grapefruit rind.

While all the pectin analyses are reported on the basis of dry weight, the same situation existed when the pectic contents were expressed on the basis of fresh weight.

#### NARINGIN

The behavior of naringin in mature or nearly mature grapefruit which remains attached to the tree does not follow a definite seasonal trend. In the fruit from Oasis the naringin content of the stem-end albedo<sup>2</sup> followed roughly the mean temperatures for the 5-day period preceding picking, with

<sup>2</sup> Naringin was not determined in the flavedo because the pigments in that tissue interfered with measurements by the colorimetric method used.

the minimum at midseason. However, the naringin minimum occurred at the second picking while the minimum temperature occurred at the time of the third picking (fig. 2). A similar temperature relationship was reported previously for the season of 1933-34 (6).

The naringin content of the albedo of fruit from Corona similarly followed roughly the changes in the mean temperature before picking. In this instance there was an increase until the end of the season (fig. 3). This seasonal trend is opposite to that recorded previously for fruit of another season from the same grove, but the temperature relationship is the same as was obtained during that season.

It will be noted in figures 2 and 3 that there is a relationship between the naringin content and the susceptibility to storage pitting. It does not follow that the presence of naringin influenced the fruit in its susceptibility to pitting but merely indicates, as was stated in the discussion of pectins, that changes take place in the composition of the fruit while it is still attached to the tree, and that these changes influence the susceptibility of the fruit to storage pitting.

The naringin content of the albedo of grapefruit from Woodlake was lowest at the time of the first picking (December 4), increased to a maximum at the time of the fourth (April 8), and subsequently decreased until the end of the season (July 2). The naringin content of the albedo of Woodlake fruit did not vary according to the temperatures preceding picking, neither did its variations coincide with those in pectic content.

In an earlier paper the fact was mentioned that the change in naringin content of stored grapefruit was controlled by factors that were poorly understood and that it might increase or decrease depending upon the condition of the fruit as well as on the conditions of storage. Present work corroborates this point of view. It also indicates a preponderant tendency for the naringin content to increase in storage through a wide range of storage temperatures with a tendency for a greater increase in fruit held at

TABLE IV

NARINGIN CONTENT OF STEM-END ALBEDO OF FRESH AND STORED GRAPEFRUIT FROM OASIS, CORONA, AND WOODLAKE, CALIFORNIA

LOCATION	No STORAGE	STORAGE			
		68° F.	56° F.	46° F.	38° F.
	%	%	%	%	%
Oasis (dry weight basis) .....	10.25	.....	12.17	11.85	11.26
Oasis (fresh weight basis) .....	2.36	.....	2.71	2.60	2.52
Corona (dry weight basis) .....	10.44	12.10	11.88	11.40	11.19
Corona (fresh weight basis) .....	2.05	2.31	2.34	2.24	2.22
Woodlake (dry weight basis) .....	8.03	.....	9.60	8.94	8.74
Woodlake (fresh weight basis) .....	1.64	.....	1.90	1.76	1.70

the higher temperatures. The average values for fruit stored 6 weeks at the various temperatures are given in table IV.

The differences are not great, especially when expressed on the fresh weight basis, but the same is true whether the percentages are expressed on the fresh or the dry weight basis.

The results reported in this paper give support to the suggestion made in the earlier report that naringin might be an indirect product of respiration, as both in the field and in storage there was a tendency for the naringin content to be higher following exposure to higher temperatures.

### Summary

1. Results are presented on the effect of season and storage temperature on the pitting developed by Marsh grapefruit and on certain chemical changes taking place in the rind of the fruit. Fruit for the study was grown in three widely separated citrus districts in California: (1) Oasis, in the Coachella Valley in the interior portion of southern California; (2) Corona, in the western portion of southern California; and (3) Woodlake, in the San Joaquin Valley.

The storage temperatures used were 38°, 46°, and 56° F. Pitting was always most severe in fruit stored at 38° and least in that stored at 56° F.

Fruit from Oasis pitted most severely at the beginning and the end of the harvest season, the severity varying directly with the mean temperature for the five days preceding picking.

Fruit from Corona pitted with varying degrees of intensity during the season, but the pitting held approximately the same relationship to the mean temperatures before picking as that from Oasis.

Fruit from Woodlake was highly resistant to pitting early in the season and became progressively more susceptible as the season advanced. There was some relationship between susceptibility to pitting and the mean temperatures preceding picking but not as marked as with fruit from Oasis and Corona.

2. Respiration of the fruit in sealed chambers was measured by the duration of the period of negative pressure. There was a direct relation between the length of this period and the susceptibility of the fruit to pitting. This relation was found to obtain in comparing fruit picked from a given group of trees in a grove, but not in comparing fruit from groves in widely separated regions.

The duration of the period of negative pressure developed by stored fruit varied according to the temperature and duration of the storage, both low temperatures and long storage periods tending to shorten the period of negative pressure. Pitted fruit had a shorter period of negative pressure than unpitted fruit from the same lot.

3. Soluble pectin in the albedo increased during the season.

Changes in protopectin did not follow a seasonal trend but there was a tendency for the minimum to occur at midseason. The changes were apparently influenced by the climatic environment.

Total pectic substances were lowest at midseason in fruit from Oasis and Corona and early in the season in the albedo of fruit from Woodlake. The variations in the albedo were such as to coincide with susceptibility to storage pitting, low pectic substances occurring at the time of low susceptibility, but no causal relationship is postulated.

The total pectic content of the albedo is somewhat higher than that of the flavedo.

Storage of grapefruit for 6 weeks did not change the quantitative relationship between the soluble pectin and protopectin.

There was an apparent slight increase of pectic substances in both the flavedo and the albedo of stored grapefruit.

4. Naringin in the albedo of grapefruit from the field varied in much the same way as the total pectic substances.

In storage naringin usually increased, and the increase was greatest at the higher temperatures.

UNITED STATES DEPARTMENT OF AGRICULTURE  
POMONA, CALIFORNIA

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# AUXIN PRODUCTION IN SEEDLINGS OF DWARF MAIZE<sup>1</sup>

J. VAN OVERBEEK

(WITH EIGHT FIGURES)

## Introduction

Dwarf races of corn are mainly characterized by the length of stem which is, at vegetative maturity, less than 50 per cent. of the length of the normal sibs. It is known that the growth in length of stems, and more especially the elongation of the cells, is regulated by auxin (11). In an earlier paper on dwarfism (8) I stated that *nana* corn, one of the genetic types of dwarfs, shows its dwarfism even in the seedling stage by its reduced growth of the mesocotyl. It was shown that this reduced growth was attributable to inhibited elongation rather than to a reduced number of cells. Recently ABBE (1) showed that in mature plants of *nana* and *dwarf-1* the number of internodes is the same as in their normal sibs. It is the shorter length of the cells in the ground parenchyma, as well as in the vascular tissue, which accounts for the short stems of the dwarfs. It was demonstrated (8) that 5 days after they were sown in sand in a physiological darkroom, *nana* plants produce less auxin than do normal ones. It was also shown that the sensitivity to auxin (measured as auxin curvatures) is less in the *nana* coleoptiles than in the normal ones. Both differences were attributed to the greater destruction of auxin in the *nana* plants, which was found to be especially high in the mesocotyls. The destruction was thought to be caused by the action of peroxidases, which were found to be much more active in the apical part of the mesocotyl of *nana* plants than of normal corn.

In 1936 a few studies on other plants appeared in which an attempt was made to link dwarfism with auxin. HINDERER (6) showed that hybrids of *Epilobium* have an auxin production which is smaller the more dwarfed the hybrid is. DE HAAN and GORTER (4) studied a tall and a shorter variety of *Pisum* and concluded that the destruction of auxin is the determining factor for the growth of the pea stem, thereby reaching the same conclusion as I had reached for the *nana* dwarf in corn. (8).

The purpose of the present investigation was to study the auxin production in several of the known genetic dwarf races of corn. In order to have comparable controls, seeds were used which segregated either 50 or 25 per cent. dwarfs. The dwarfs of the types here described were easily distinguished from normal control plants 4 days after they had been sown in the darkroom. The seeds were sown and the plants were grown in pure sand (without nutrient solution). The experiments were carried out in the same darkroom where the plants were grown. The temperature was 24° C. and

<sup>1</sup> Second communication on the physiological basis of dwarfism.

the humidity 90 per cent. The plants were occasionally exposed to the orange light of the darkroom lamps.

### Experimentation

#### FACTORS AFFECTING THE AMOUNT OF AUXIN GIVEN OFF BY COLEOPTILE TIPS

The production of auxin was determined by the diffusion method. The tips of the coleoptiles were cut off and placed on agar blocks. The auxin produced in the tips was given off into the agar. The auxin content of the agar was then determined by means of the standard *Avena* test (3, 10, 11). The yield of auxin obtained in this way is dependent upon the following conditions.

(a) RIDDING CUT SURFACE OF DESTRUCTIVE ENZYMES.—It was found that the yield of auxin could be markedly increased by standing the tips on wet filter paper before putting them on the agar blocks. Coleoptile tips were placed on wet filter paper for 0,  $\frac{1}{2}$ , 1, and  $1\frac{1}{2}$  hours before they were put on

TABLE I

AMOUNT OF AUXIN (IN DEGREES OF CURVATURE) DIFFUSED FROM COLEOPTILE TIPS INTO AGAR BLOCKS. AFTER THE TIPS WERE CUT THEY WERE PLACED ON WET FILTER PAPER BEFORE BEING PUT ON THE AGAR BLOCKS. NORMAL CORN. 60529

TIME ON WET FILTER PAPER	AGE OF CORN SEEDLING IN DAYS		
	4	6	7
hr.			
0 .....	4.8*	6.8	3.4
$\frac{1}{2}$ .....	5.0	8.3	9.7
1 .....	7.4	12.0	8.7
$1\frac{1}{2}$ .....	7.5	12.3	9.0

\* Averages of 12 plants, tips, etc. The same holds for the other tables and graphs unless the contrary is stated.

agar blocks (table I). It is clear that the yield increased with the time the cut surface was "washed." The maximum effect is reached by standing for 1 hour on wet filter paper. The effect of standing on wet filter paper is readily explained by assuming that by this treatment the cut surface is cleared from enzymes having a destructive action on auxin. It was shown (9) that a cut surface, after it had been in contact with wet filter paper for some time, had a much lower peroxidase activity than a freshly cut surface. In most experiments described in this paper the coleoptiles were put for 1 hour on wet filter paper before they were placed on the agar blocks.

(b) DURATION OF AUXIN PRODUCTION.—Isolated coleoptile tips are able to give off auxin at the same rate for at least several hours. Table II shows

TABLE II

AMOUNT OF AUXIN (IN DEGREES OF CURVATURE) GIVEN OFF BY 5-MM. TIPS OF 5-DAY-OLD COLEOPTILES. THE TIPS WERE PLACED ON FRESH AGAR BLOCKS EVERY 45 MINUTES. 60309

VARIETY OF CORN	SUCCESSIVE PERIODS OF 45 MINUTES					
	1	2	3	4	5	6
Normal .....	15.5	17.0	16.0	18.0	16.0	13.1
<i>Nana</i> .....	9.0	9.2	10.6	7.6	8.7	8.7

the results of an experiment with tips of 5-day-old *nana* and normal plants. The tips were placed on fresh agar blocks each 45 minutes for 5 successive times. The amounts of auxin diffusing into the blocks were practically constant during the time of the experiment.

(c) LENGTH OF THE TIP.—The length of the tip does not greatly affect the amount of auxin given off. The 5-mm. tips used in the experiment recorded in table II continued to give off auxin at the same rate for  $4\frac{1}{2}$  hours. In table III results of two experiments with tips 1 and 2 mm. long are presented. These short tips, too, continued to give off auxin for  $4\frac{1}{2}$  to 6 hours at practically the same rate. These experiments show that the production of auxin by the extreme tip, when isolated, may continue for a considerable time.

TABLE III

AMOUNT OF AUXIN (IN DEGREES OF CURVATURE) GIVEN OFF BY COLEOPTILE TIPS OF ABOUT 5-DAY-OLD SEEDLINGS DURING SUCCESSIVE PERIODS OF  $1\frac{1}{2}$  HOUR

VARIETY OF CORN	LENGTH OF TIP	EXPERIMENT NUMBER	SUCCESSIVE PERIODS OF $1\frac{1}{2}$ HR.			
			1	2	3	4
Normal .....	2 mm.	41024	18.6	17.1	19.0	
<i>Nana</i> .....	2 mm.		11.0	13.5	15.9	
Normal .....	1 mm.	41025	15.0	6.0	11.0	12.0
<i>Nana</i> .....	1 mm.		8.0	7.0	6.0	7.0

(d) AGE OF THE SEEDLING.—The age of the seedling is an important factor in determining the amount of auxin given off by the coleoptile tips. In figure 1 the age of the seedling (days after the seeds were planted in the sand) is plotted against the production of auxin. The length of the tips was 5 mm., a length which was maintained for most of the tips used in experiments described in this paper. The tips were placed for 1 hour on the agar blocks. The amount of auxin given off is expressed in degrees of curvature obtained in the standard *Avena* test. A control test with blocks con-

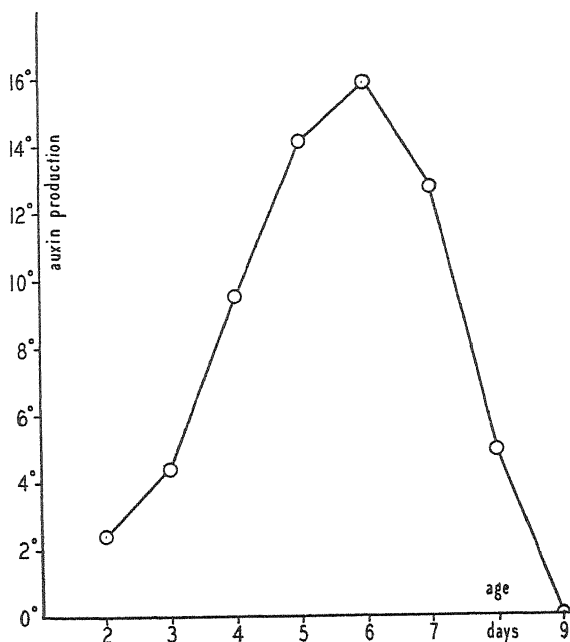


FIG. 1. The relation between the auxin production of the coleoptile tip and the age of the seedling in a normal corn strain (60316). At the stage of optimal production an amount of  $2 \times 10^{-10}$  gram of auxin per hour per tip is given off.

taining a known concentration of indole-acetic acid showed that a curvature of  $8.9^\circ$  was obtained if the concentration in the agar blocks was equivalent to 64.5 gamma per liter. The blocks on which the corn tips had been standing contained auxin-a or auxin-b, which are according to KÖGL and KOSTERMANS (7), twice as active by weight as indole-acetic acid. Hence, the concentration of auxin-a or auxin-b in the block on which one tip of a 4-day-old coleoptile (figure 1) had been standing for one hour was about 34 gamma per liter. Since the agar block on which one tip was standing had a volume of 4 mm.<sup>3</sup>, the total amount of auxin-a or auxin-b given off per hour by one tip of a 4-day-old normal corn seedling was  $1.36 \times 10^{-4}$  gamma ( $1.36 \times 10^{-10}$  gm.). In corn seedlings the primary leaf will break through the coleoptile on about the fifth day. This means that the auxin production is still large and has not even reached its optimum when the leaf breaks through.

#### THE AUXIN PRODUCTION OF NANA SEEDLINGS

The *nana* character is inherited as a simple, recessive Mendelian character. The gene is located in the third chromosome. The gene locations are quoted after EMERSON, BEADLE, and FRASER (5).

(a) THE AUXIN CONTENT OF THE SEED.—Large amounts of auxin can be

extracted from corn seeds. The question arose whether seeds producing *nana* plants differ in auxin content from seeds producing normal ones. This was worked out in the following way. Seeds were obtained by back-crossing pure *nana* dwarfs with an  $F_1$  plant. Such seeds will segregate 50 per cent. *nana* plants and 50 per cent. normal ones. The individual grains were ground in a mortar (average weight of 1 seed was 0.125 gm.). The pulverized seed was extracted with 5 cc. of distilled water for about 15 hours at 2 to 3° C. An agar block was soaked in the extract. The agar block was analyzed by means of the standard *Avena* test. The results of these determinations are plotted in figure 2. The auxin content of the seeds

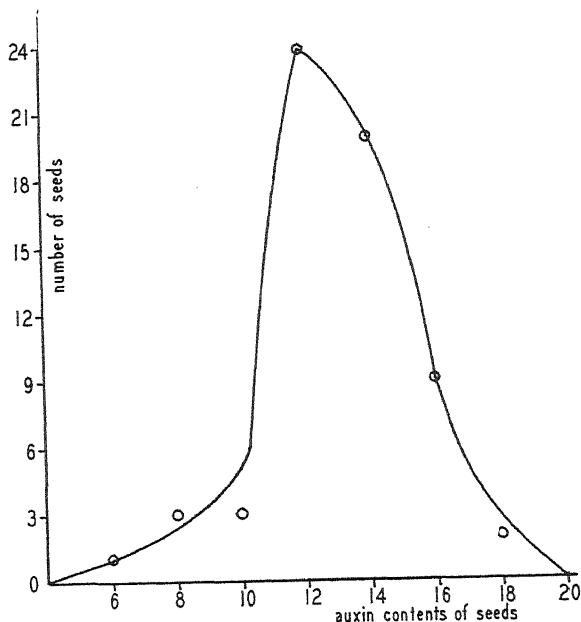


FIG. 2. Frequency curve of the auxin content of individual corn grains of a mixture consisting of 50 per cent. *nana* seeds and 50 per cent. of normal seeds (60114).

was plotted on the abscissa and the number of seeds having a certain auxin content is plotted on the ordinate. The resulting curve is a frequency curve having one single peak, indicating that the *nana* seeds do not differ from the normal ones in regard to auxin content. A control test with other seeds of the same sample showed that 50 per cent. of the germinated seeds developed into dwarfs and the remaining ones into normal corn plants, as could be expected.

(b) PRODUCTION AND GROWTH CURVES.—The production of auxin of the dwarfs has been determined in seedlings from 4 to 7 days old. The lower limit was set because it was practically impossible to distinguish dwarfs

from normal plants at a stage earlier than 4 days after the seeds had been planted. The higher limit of 7 days was chosen because it was shown (figure 1) that the production after this day decreases rapidly. Figure 3a

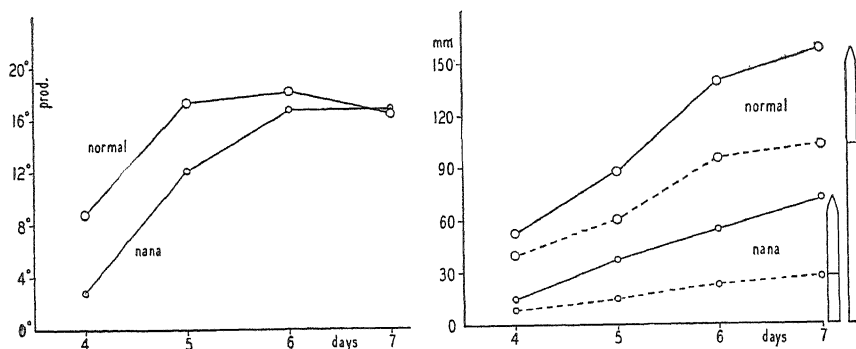


FIG. 3a. Left. Relation between the amount of auxin given off by the coleoptile and the age of the seedling in series segregating *nana* dwarfs. Ordinate: amount of auxin given off by 12 tips, of 5 mm. length, per hour. The amount is expressed in degrees of curvatures in the *Avena* test. Abscissa: age of the seedlings counted from the moment the seeds were planted in sand. Averages of 48 plants (60316, 60502, 60514, 60515).

FIG. 3b. Right. The length of *Nana* plants as compared with normal ones at various stages of development. The dotted lines indicate the position of the coleoptilar nodes, which separate the coleoptile and the mesocotyl.

shows that the production of auxin by *nana* dwarfs is considerably lower than that of the normal sib when the plants are 4 or 5 days old. In older plants, however, the auxin production is practically the same for *nana* and normal plants. The length of the dwarfs and that of the normal plants at the various stages of development are given in figure 3b. It is especially the mesocotyl which grows much more slowly in the *nana* plants than in the normal ones. The fact that the production of auxin in the coleoptile tip of 6- and 7-day-old *nana* plants is the same as that in normal ones does not necessarily mean that the growing zones receive equal amounts of auxin.

TABLE IV

AMOUNTS OF AUXIN GIVEN OFF AT THREE DIFFERENT LEVELS OF CORN SEEDLINGS. PLANTS 6 DAYS OLD. CUT SURFACES WASHED IN WATER FOR 1½ HOURS PRIOR TO DIFFUSION TEST. 60319

Cut surface 5 mm. below coleoptile tip .....	Normal 49.0°
	<i>Nana</i> 46.6° ( 5% less than normal)
Cut surface 2 mm. above coleoptilar node	Normal 22.3°
	<i>Nana</i> 17.5° (22% less than normal)
Cut surface 2 mm. below the node .....	Normal 11.6°
	<i>Nana</i> 1.5° (87% less than normal)

This is shown by the following experiments. Six-day-old plants were divided into three groups. From the plants of the first group (see table IV) 5-mm. coleoptile tips were cut, and the amount of auxin given off by them was determined. It was found that the *nana* plants of this set produced about 5 per cent. less auxin than the normal controls. In the next set the plants were cut off 2 mm. above the node, and by placing agar blocks against the cut surface it could be determined how much auxin, approximately, would reach the basal region of the coleoptile. It was found that in this region the *nana* plants received 22 per cent. less auxin than the normal ones. The plants of the third set were cut off 2 mm. below the coleoptilar node, and the amount of auxin diffusing out of the cut surface was collected in agar. It was found that in the region below the coleoptilar node, which is the growing region of the mesocotyl, 87 per cent. less auxin was present in the dwarfs than in the normal plants. The way the auxin travels from the coleoptile tip to the mesocotyl is for 6-day-old plants shorter for the dwarfs than for the normal plants as follows from figure 3b. Hence, this can not account for the greater disappearance of auxin in the *nana* plants. In a previous paper on *nana* dwarfs it was concluded that the greater destruction in *nana* plants was responsible for their reduced growth. This destruction was determined again for the 6-day-old plants. Sections were cut from the basal part of the coleoptiles in such a way that the basal cut surface was just at the coleoptilar node. Such sections were placed for 3 hours on wet filter paper in order to free them of the auxin they might contain. After that period of time they were placed with their basal cut-surface on agar blocks containing 16.9°-indole-acetic acid. After 1½ hours the sections were removed and the amount of auxin remaining in the blocks determined. It was shown that 37 per cent. of the auxin was destroyed by the sections of normal corn plants, whereas 92 per cent. was destroyed by the sections of the dwarfs (table V). These facts support the conclusion previously

TABLE V

DESTRUCTION OF AUXIN BY 6-DAY-OLD *nana* AND NORMAL CORN PLANTS. SECTIONS 5 MM. LONG WERE CUT OUT OF THE BASAL PART OF THE COLEOPTILE. THE LOWER CUT WAS THROUGH THE COLEOPTILAR NODE. THE SECTIONS WERE PUT WITH THEIR MORPHOLOGICAL BASE ON WET FILTER PAPER FOR 3 HOURS FOLLOWED BY 1½ HOURS ON AGAR BLOCKS CONTAINING AUXIN. AVERAGES OF 48 TO 24 PLANTS. 60605

Initial auxin concentration in the agar blocks .....	16.9°
Left by section of normal corn .....	10.7° (destroyed 37%)
Left by sections of <i>nana</i> corn .....	1.4° (destroyed 92%)

reached (7) that in the *nana* dwarfs the excessive destruction of auxin is responsible for their reduced growth.

## AUXIN PRODUCTION AND GROWTH OF DWARF-1

Figures 4a and 4b present a similar set of curves for *dwarf-1* as the figures 3a and 3b for *nana*. The auxin production of *dwarf-1* remains below

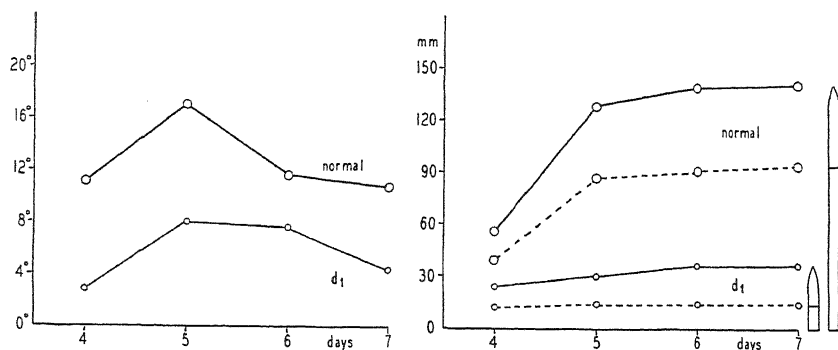


FIG. 4a. Left. Relation between the amount of auxin given off by the coleoptile and the age of the seedling in series segregating dwarf-1. Ordinate and abscissa as in figure 3a. Average values of 36 plants (60415, 60511, 60519).

FIG. 4b. Right. The length of dwarf-1 as compared with normal plants at various stages of development.

that of the normal plant at all stages of development. *Dwarf-1* is a much more extreme dwarf than is *nana*, as well in mature as in seedling stage. Both the coleoptile and the mesocotyl are much shorter in the dwarf than in the normal control plants. The location of the *d<sub>1</sub>* gene is in the third chromosome as in *nana*, but the genes are located at opposite ends of the chromosome.

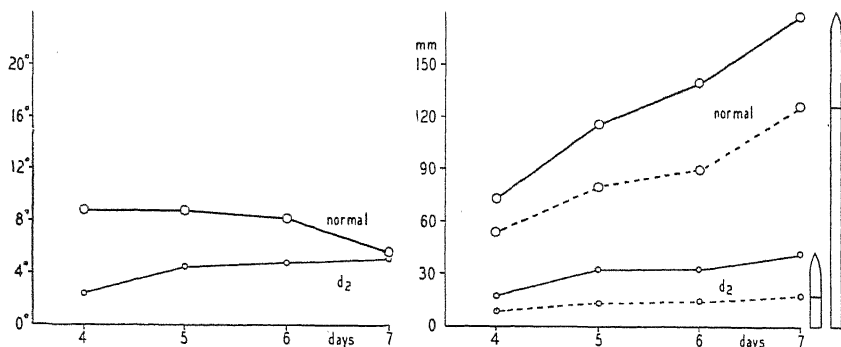


FIG. 5a. Left. Relation between the amount of auxin given off by the coleoptile and the age of the seedling in series segregating dwarf-2. Ordinate and abscissa as in figure 3a. Average values of 36 plants (60408, 60430, 60521).

FIG. 5b. Right. The length of dwarf-2 as compared with normal plants at various stages of development.



## AUXIN PRODUCTION AND GROWTH OF DWARF-2

*Dwarf-2* is very similar in appearance to *dwarf-1*. The strain used in the experiments had a poor viability. The normal plants were very thin and they grew faster in the early stages of development than does the average corn seedling. In the figures 5a and 5b it is shown that the auxin production of the dwarf is lower than that of the normal plant. The location of the  $d_2$  gene is in the third chromosome.

## AUXIN PRODUCTION AND GROWTH OF DWARF-3

*Dwarf-3* is also an extreme dwarf like the *dwarf-1* and *dwarf-2*. The stock used was a vigorous one. This dwarf, too, had an auxin production lower than that of the normal sib at all stages of development. The curves shown in figures 6a and 6b are similar to those of *dwarf-1*. The gene is located in chromosome 9.

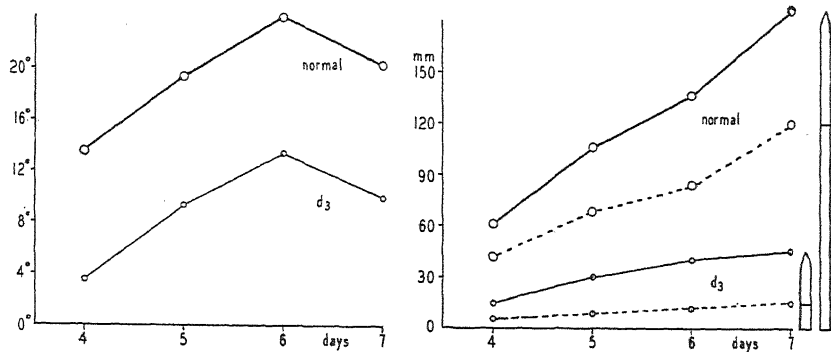


FIG. 6a. Left. Relation between the amount of auxin given off by the coleoptile and the age of the seedling in series segregating dwarf-3. Ordinate and abscissa as in figure 3a. Averages of 36 plants (60520, 60526, 60527).

FIG. 6b. Right. The length of dwarf-3 as compared with normal plants at various stages of development.

## AUXIN PRODUCTION AND GROWTH OF DWARF-7

At seedling stage *dwarf-7* is very similar in appearance to *dwarf-1* and the other extreme dwarfs. At vegetative maturity, however, this dwarf is much taller than the other dwarfs, including *nana*, and may reach more than half the length of the plants of the normal sib. Figures 7a and 7b are the growth and auxin production curves for *dwarf-7*, whose gene  $d_7$  is located in chromosome 10.

## AUXIN PRODUCTION AND GROWTH OF PIGMY

The *pigmy* dwarf is one of quite different type than the ones discussed above. In the field it is characterized by its short and striated leaves rather

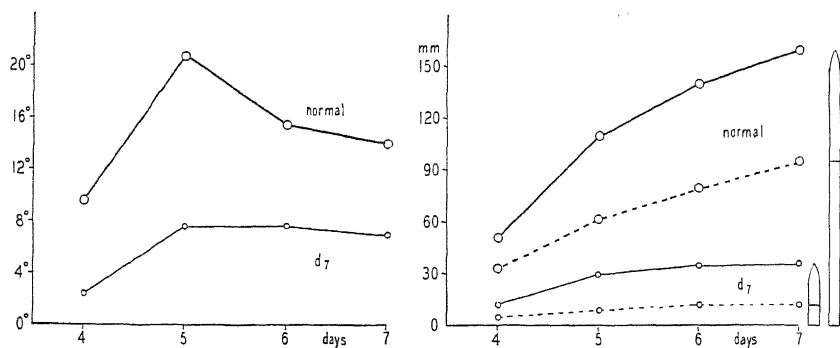


FIG. 7a. Left. Relation between the amount of auxin given off by the coleoptile and the age of the seedling in series segregating dwarf-7. Ordinate and abscissa as in figure 3a. Average values of 36 plants (60429, 60522, 60525).

FIG. 7b. Right. The length of dwarf-7 as compared with normal plants at various stages of development.

than by its short internodes. In the seedling stage the coleoptile is extremely small, whereas the length of the mesocotyl is of the type of *nana*. The small size of the leaves and the coleoptile may be correlated, since the coleoptile of grasses is considered to be a leaf (see 2). The auxin production of pigmy plants is lower than in the normal ones. The type of curve resembles rather that of *nana* than those of the extreme dwarfs (fig. 8 a and b). The gene *py* is located in chromosome 6.

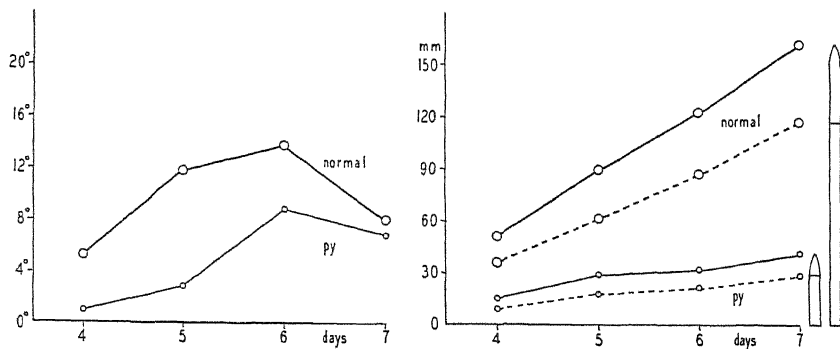


FIG. 8a. Left. Relation between the amount of auxin given off by the coleoptile and the age of the seedling in series segregating pigmy dwarfs. Ordinate and abscissa as in figure 3a. Averages of 36 plants (60513, 60512, 60427).

FIG. 8b. Right. The length of pigmy plants as compared with normal ones at various stages of development.

### Summary

1. The amount of auxin given off by the coleoptile tips of etiolated maize seedlings was studied. Washing of the cut surface increases the amount of auxin diffusing out of these tips into agar blocks (table I).

2. The tips continue to give off auxin at approximately a constant rate for several hours after they are cut off (table II).

3. Isolated tips as short as 1 mm. continued to give off auxin for more than six hours at only slightly reduced rate at the end of that period (table III).

4. The production of auxin varies with the age of the seedlings. On the fifth to the sixth day after sowing, the production is optimal (fig. 1). At this stage about  $2 \times 10^{-10}$  gram of auxin per tip per hour is given off.

5. Seed producing *nana* dwarfs were found to contain the same amount of auxin as seed producing normal plants (fig. 2).

6. When 4 and 5 days old, *nana* dwarfs produce less auxin than do normal plants; when 6 and 7 days old, they produce equal amounts (fig. 3). The amount of auxin reaching the growing zones of the 6- and 7-day-old *nana* plants is much smaller, however, than in the normal plants (table IV). This is caused by the greater destruction of auxin in the *nana* dwarfs (table V).

7. The production of auxin in *dwarf-1*, *dwarf-2*, *dwarf-3*, *dwarf-7*, and the *pigmy* dwarf was found to be lower than that of their normal sibs (figs. 4 to 8).

WILLIAM G. KERCKHOFF LABORATORIES  
CALIFORNIA INSTITUTE OF TECHNOLOGY  
PASADENA, CALIFORNIA

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# CORRELATION BETWEEN GROWTH OF EXCISED ROOT TIPS AND TYPES OF FOOD STORED IN THE SEED

GLADYS C. GALLIGAR

(WITH TWO FIGURES)

## Introduction

Tissue culture had its inception near the close of last century (2), and during the past thirty-five years the cultivation of animal tissues has progressed markedly, while attempts to grow several types of plant tissue have met with varying degrees of success. The first reports on the growth of excised root tips were published by ROBBINS (7) and KOTTE (4) about twelve years ago. The object of the present investigation was to determine whether any correlation exists between the type of stored food in a given seed and the behavior of its excised root tip in sterile nutrient solution.

## Materials and methods

Root tips from representative seeds containing high percentages of oil, sugar, starch, and protein, respectively, were selected to study their growth behavior when left undisturbed to grow to as great length as possible under the conditions imposed. Sunflower (*Helianthus annuus*), cotton (*Gossypium barbadense*), and castor bean (*Ricinus zanzibarensis*) were chosen for high oil content; Gradus wrinkled pea (*Pisum sativum*) and sweet corn (*Zea mays*) for high sugar content; Reid's Yellow Dent corn (*Zea mays*) and Hopi Indian corn (*Zea mays*) for starch; and Burpee's Extra Early smooth pea (*Pisum sativum*) and Manchú soybean (*Soja hispida*) for high reserves of protein.

The procedure to secure sterile root tips involved several steps and in the main followed that of ROBBINS (7, 8). The seeds were soaked for 4 hours in a solution of zonite diluted 1:30. They were then transferred by means of steel forceps to petri dishes containing a thin layer of 0.75 per cent. sterile agar, where they were allowed to germinate. The ends of the forceps were passed through a Bunsen flame and cooled in sterile water each time before touching the seeds. When the roots had grown approximately 1 inch, the tips were cut off in uniform lengths of 10 mm. by means of a steel scalpel, which was flamed and cooled before each operation. The excised root fragments were transferred with a sterilized wire loop to pyrex Erlenmeyer flasks of 125-ml. capacity carrying 50 ml. of sterile nutrient media. The flasks were kept in a laboratory with curtains drawn, where they received very weak diffuse light and where the temperature varied no more than 3° F. (68–71° F.) during the experiment.

The nutrient solution was a modification of Pfeffer's formula to which was added dextrose and peptone.<sup>1</sup> Immediately after being made, the solution was measured into the flasks, which were closed with cotton plugs and autoclaved at 15 lb. pressure for 20 minutes.

Germination was 90 to 100 per cent. Reid's Yellow Dent corn and the soybeans were secured from the Agriculture Experiment Station, University of Illinois. The Hopi corn was obtained from Indian traders in Arizona. All other seeds were ordered from the Burpee Seed Company.

From each species or variety 40 root tips were grown, with the exception of castor bean and soybean, from which sterile root tips were difficult to obtain. Five to ten individuals were grown in successive replicates, to avoid possible errors in growing only one series of 40 each. The cultures were maintained so long as growth was apparent as evidenced by noticeable elongation; some individuals, however, were left beyond this period with rather surprising results.

Attempts were made to measure increments of elongation at 12- and 24-hour intervals by means of a millimeter rule placed under the flask. The termination of the period of measuring was conditioned by the time required for the root tip to curve within the flask, which usually occurred on the tenth day with rapidly growing root tips.

The experiments were performed in the physiological laboratories at the University of Illinois.

### Discussion of results

In every case except castor bean, table I shows that daily increments of length in individuals rise and decline sharply and irregularly with no evidence of a smooth sigmoid growth curve. This behavior may be caused by recovery from wound shock at the point of excision, or individual differences, or repeated adjustment to an artificial medium. The root tips were removed from their normal source of nutriment for growth and development to an artificial medium, from which they absorbed, perhaps rather poorly at best, the foods used in their metabolism. There can be no certain prediction of the behavior of protoplasm when it is subjected to a given set of external conditions. There is always the internal adjustment to the external environment, and this may be as variable as the number of individuals. Since the excised root tips used in this study behaved so differently and since no figures are available to demonstrate that a medium has been found with an optimal environmental complex perfectly adapted to the growth of isolated root tips of any variety or species, it is at present a great error to attach any practical significance to the results obtained. Until a medium is devel-

<sup>1</sup> Ca(NO <sub>3</sub> ) <sub>2</sub>	2.0 gm.	KCl	0.25 gm.	Dextrose	2.0 per cent.
KH <sub>2</sub> PO <sub>4</sub>	0.5 "	MgSO <sub>4</sub>	0.5 "	Peptone	0.04 per cent.
KNO <sub>3</sub>	0.5 "	FeCl <sub>3</sub>	0.005 "	Distilled H <sub>2</sub> O	6000 cc.

TABLE I

SAMPLE SERIES OF FIVE INDIVIDUALS OF EACH SPECIES SHOWING DAILY INCREMENTS OF LENGTH IN MILLIMETERS FOR EACH INDIVIDUAL DURING THE MEASURABLE STAGE

Individuals	Daily increment in millimeters														
	Sunflower														
1 .....	2	3	3	0	9	6	5	3	0	2	2	3	...	...	...
2 .....	1	0	5	2	0	3	5	3	1	1	0	2	...	...	...
3 .....	0	0	8	2	4	5	6	1	0	0	0	0	...	...	...
4 .....	3	5	7	0	7	8	10	8	5	5	...	...	...	...	...
5 .....	3	0	4	1	0	2	3	1	1	1	1	1	...	...	...
	Cotton														
1 .....	3	3	3	5	2	0	2	2	0	0	1	...	...	...	...
2 .....	2	1	2	5	2	3	12	2	0	3	2	2	2	...	...
3 .....	2	2	1	3	3	0	0	0	0	1	1	0	0	...	...
4 .....	2	0	3	7	2	1	0	1	0	0	0	1	0	...	...
5 .....	2	0	0	1	1	2	3	4	0	0	1	...	...	...	...
	Castor Bean														
1 .....	5	0	0	1	1	0	0	0	0	0	0	...	...	...	...
2 .....	5	1	1	1	0	0	0	0	1	0	0	...	...	...	...
3 .....	5	2	1	0	0	0	0	0	0	0	0	...	...	...	...
4 .....	4	2	2	1	0	0	0	1	0	0	0	...	...	...	...
5 .....	5	2	1	1	1	0	0	0	0	0	0	...	...	...	...
	Corn														
1 .....	3	1	5	14	4	5	6	6	7	5	...	...	...	...	...
2 .....	13	7	0	3	5	10	3	2	5	5	...	...	...	...	...
3 .....	4	4	2	5	6	10	2	3	2	3	0	2	5	...	...
4 .....	3	2	2	3	7	3	2	4	4	3	3	0	5	...	...
5 .....	5	0	7	13	0	5	7	5	5	5	1	3	5	...	...
	Hopi Indian corn														
1 .....	7	3	3	5	5	5	5	5	3	2	2	3	4	4	2
2 .....	0	3	6	10	1	0	4	4	0	0	3	3	2	2	1
3 .....	1	2	4	4	5	0	13	5	4	3	1	3	3	1	2
4 .....	0	3	5	7	0	0	0	10	5	3	2	5	4	3	5
5 .....	1	1	1	0	2	0	3	3	0	2	1	0	2	2	2
	Gradus pea														
1 .....	2	1	2	2	2	3	0	0	0	0	2	0	0	...	...
2 .....	5	3	2	4	1	0	0	0	0	0	0	0	0	...	...
3 .....	2	1	2	1	1	2	0	0	0	0	2	0	0	...	...
4 .....	2	4	1	4	4	4	1	0	0	2	0	0	0	...	...
5 .....	3	2	2	3	3	3	1	0	0	0	0	0	0	...	...
	Sweet corn														
1 .....	2	3	3	0	9	7	5	3	0	2	2	3	...	...	...
2 .....	1	0	5	0	2	3	5	3	1	1	0	2	...	...	...
3 .....	0	0	8	2	4	5	6	1	0	0	0	0	...	...	...
4 .....	3	5	7	0	7	8	10	8	5	5	...	...	...	...	...
5 .....	3	0	4	0	1	2	3	1	1	1	1	1	...	...	...

TABLE I (*Continued*)

Individuals	Daily increment in millimeters													
	Burpee's Extra Early Pea													
1 .....	2	0	0	0	1	0	0	0	0	0	0	0	...	...
2 .....	3	0	0	0	0	0	0	0	0	0	0	0	...	...
3 .....	5	10	2	6	5	7	0	0	0	0	0	0	...	...
4 .....	2	0	1	1	0	1	0	0	0	0	0	0	...	...
5 .....	1	0	2	0	0	0	1	1	0	0	0	0	...	...
	Soybean													
1 .....	0	0	0	0	0	0	0	0	0	0	0	0	...	...
2 .....	0	0	0	0	0	0	0	0	0	0	0	0	...	...
3 .....	1	1	0	1	0	0	0	0	0	0	0	0	...	...
4 .....	1	0	1	1	0	0	0	0	0	0	0	0	...	...
5 .....	2	1	1	1	0	0	0	0	0	0	0	0	...	...

oped in which the excised root tips respond similarly in their growth and development to their habits under natural conditions, it is hardly possible to work out any accurate usable knowledge in regard to their fundamental metabolism. Previous and present studies, however, are paving the way for subsequent work on this subject.

For a 10-day period the average daily rate of elongation of corn surpassed that of other root tips under observation (table II). Sweet corn and Hopi corn were approximately equal in their average daily growth and about three-fourths as rapid as dent corn. Next in order was cotton, rather closely followed by sunflower, both growing about one-half as rapidly as corn; Gradus pea exhibited a rate of about one-fourth, castor bean about one-fifth, and Burpee's Extra Early pea about one-eleventh the rate of dent corn. Root tips from soybean made little growth within the 10-day period. The greatest growth on the first day was made by an individual root tip of dent corn which increased 13 mm., a gain of 130 per cent. over the original length. The greatest increment achieved by any root tip on any day within the 10-day period was 14 mm., also made by dent corn. It is difficult to give accurate data concerning the rapidity of elongation after the measurable stage was passed, but certainly in some cases a marked growth was observed.

Dry weight determinations and final-length figures on the roots reveal some interesting contrasts to the daily rates of growth during the initial period (table II). Sunflower achieved an average length of 17.55 mm. in the 10-day period as compared to 23.6 mm. in cotton, but reached an average total length of 60.8 mm. as compared to 43 mm. in cotton, an approximate ratio of 3:2. On the other hand, the ratio of dry weights of the two is approximately 10:1, showing that cotton is less able to adapt itself to the nutrient medium, and in the process of inanition drew on more of its own stored reserves than did sunflower under the same conditions. Castor bean



TABLE II

SUMMARY OF DATA FOR 360 INDIVIDUAL ROOT TIPS CUT OFF AT ORIGINAL LENGTHS OF 10 MM. AND GROWN AS LONG AS POSSIBLE IN THE DARK IN MODIFIED PFEFFER'S SOLUTION PLUS 2 PER CENT. DEXTROSE AND 0.04 PER CENT. PENTONE

SPECIES	AV. DAILY INCREASE IN LENGTH OF 30 ROOT TIPS FOR FIRST 10 DAYS										AV. TOTAL LENGTH AT END OF 10 DAYS	AV. FINAL TOTAL LENGTH	RANGE IN FINAL LENGTHS IN MM. AMONG IN- DIVIDUALS	FINAL AV. NO. LATERAL ROOTS	RANGE IN FINAL NO. LATERAL ROOTS AMONG IN- DIVIDUALS	AV. DRY WT. PER 10 ROOTS
	1	2	3	4	5	6	7	8	9	10						
	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.						
Oily seeds	4.71	1.20	1.05	0.70	0.24	0.00	0.00	0.25	0.23	0.00	mm.	mm.	mm.	0.0	0	gm.
Castor bean	2.65	1.55	0.60	3.70	2.65	3.04	1.63	0.52	0.97	0.31	8.39	16.2	11-23	0.0	0	0.031
Sunflower ...	2.71	2.94	3.23	4.20	2.93	1.74	4.20	0.91	0.63	0.33	17.72	60.8	23-75	27.0	0-63	0.244
Cotton .....											23.82	43.0	17-75	14.0	0-28	0.025
Starchy seeds	4.81	3.15	5.70	3.92	4.35	5.00	4.21	3.27	4.89	5.77	45.07	241.4	36-1570	126.0	0-721	0.266
Corn .....																
Hopi	1.82	2.41	3.80	5.21	2.67	1.15	7.05	4.42	2.01	1.80	32.32	103.7	23-430	21.0	0-45	0.055
Indian Corn																
Seeds con- taining sugar																
Grass pea	1.86	1.95	2.20	1.65	1.67	1.64	0.73	0.62	0.32	0.25	12.89	20.2	10-53	2.0	0-8	0.034
Sweet corn	1.82	1.67	5.43	0.42	4.67	5.03	5.88	3.21	1.41	1.82	31.36	98.1	43-315	17.0	5-52	0.144
Seeds high in protein																
Burpee's																
Extra																
Early pea	1.75	1.00	0.72	0.65	0.55	0.65	0.30	0.35	0.20	0.00	6.17	22.9	10-47	0.12	0-1	0.029
Soybean ...	0.35	0.41	0.33	0.00	0.24	0.00	0.00	0.00	0.00	0.00	1.33	14.7	10-25	3.0	0-10	0.022

ceased activity and died too early for much of its own reserve to be drawn upon. It is quite obvious from the data at hand on castor bean, sunflower, and cotton that no correlation exists between oil reserves in seeds and subsequent growth behavior of excised root tips from such seeds under the conditions imposed.

Although dent corn manifested a much greater final elongation than any species used, the average dry weight was only slightly greater than that of sunflower. This indicates a greater ability on the part of the protoplasm of corn to prolong its activity in inanition by transfer of reserves in mature tissues to the actively growing meristem. It is possible, however, that the corn roots were unable to absorb as much nutriment from the medium as sunflower, and were thereby forced to draw more heavily upon their own reserves. Hopi corn and sweet corn also demonstrated an ability to maintain themselves by transfer of nutriment from their own mature tissues.

Gradus pea, Burpee's Extra Early pea, and soybean died before much of the stored reserves in the older portions of the root had been transferred to the growing tips. It is of interest to note that all of the root tips belonging to the family Leguminosae behaved poorly in comparison with the other root tips used.

In the progress of development under the conditions stated each species and variety exhibited certain peculiarities of its own, which will be described briefly in the following paragraphs.

Castor bean exhibited greatest increase in length on the first day, the rate gradually falling off until the fourth or fifth day, and showing no further increase in length except for an occasional millimeter or two on the sixth or seventh day. This species was the only one which exhibited anything like a fairly uniform growth rate. The root tips never developed laterals and never surpassed a total length of 25 mm., a gain of only 150 per cent over the original length. A grayish discoloration began on the third day, and covered the entire root by the time growth ceased.

The root tips of cotton invariably began to darken within 3 to 5 days, and, after growth stopped, turned completely black. From the ninth day onward lateral roots made their appearance, but never grew more than 13 mm. and never branched (fig. 1).

Sunflower root tips exhibited some very interesting responses. Secondary roots practically always appeared on the fifth day. Often, however, instead of producing secondary roots, there developed a series of cone-like papillae, which were approximately 2 mm. in diameter at the base and projected about 1.5 mm. from the surface of the main root (fig. 1). These papillae remained papillae in many instances; in other instances secondary roots pushed through the tops of these cones within 3 to 27 days after the formation of the papillae. Examination indicated that these elevations were

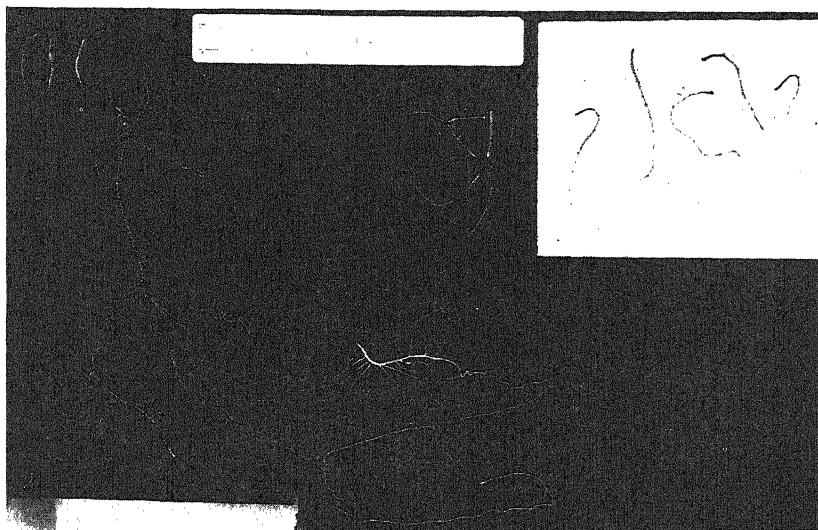


FIG. 1. Growth habits of several varieties and species of roots cut off when 10 mm. long and then allowed to grow without disturbance in Pfeffer's solution plus glucose and peptone. Left: four sunflower roots after four and one-half months' growth; the three short roots at upper left did not grow after the first month; note peculiar papillae and lateral roots developed from papillae. Upper center: a sunflower root that remained as the three in the upper left corner for 3 weeks; then produced lateral moniliform roots. Lower center: root of Hopi Indian corn, 4 months old and 430 mm. long. Upper right: cotton root tips five weeks old.

formed by secondary roots pushing against the outer two or three layers of cortical cells and the epidermis of the main axis, which did not yield to the growing tip of the secondary root for days or weeks and sometimes not at all. In the instances where the secondaries eventually emerged, they were exceedingly fine and threadlike and the papillae formed large collars around their bases. Occasionally the main axis of the root ceased elongation at 20 to 27 mm. with no secondaries growing through the characteristic papillae. On some of these individuals after a lapse of as much as two and one-half weeks, in some instances, laterals developed within 2 mm. of the tip and grew to lengths of 3 to 5 times those of the main axes with several branches of their own. This illustrates excellently that when growth in length ceases, differentiation proceeds close to the tip of the root. Many such laterals were very fine and threadlike; others of greater diameter possessed alternating regions of swelling and constriction, giving the appearance of a strand of spindle-shaped beads (fig. 1). Such moniliform roots are not unlike the peculiar constrictions secured in root tips of wheat cut at 0.1 mm. and grown in hanging drops by WHITE (9), who suggested that the constrict-

tions were due to a "hyperhydric" change, a term first used by KÜSTER (5). HORTES (3) has produced this peculiar beaded effect by dipping the tip of a growing root into cold water at daily intervals. When moniliform roots occur under natural conditions, they usually may be explained by fluctuation of water supply or by physical constriction by such agents as wire, stones, etc. It is obvious that none of the above explanations fit the present case. Since only sunflower root tips exhibited the phenomenon to any noticeable degree (figs. 1, 2), it appears to be a peculiar response of the protoplasmic activities of this species to the medium in which it was grown. Whenever a root of sunflower, as well as other roots, grew to such an extent that several of the growing points were pushed above the surface of the medium, root hairs formed in much greater abundance on the exposed parts, and were evident to within 1 mm. of the tip (fig. 1). It is common knowledge that ordinarily root hairs form less abundantly in water than in soil (1). The root tips of sunflower were translucent in the actively growing stage and turned brown with age.

Root tips of dent corn manifested a variety of forms in their growth, which was probably due to genetic differences, since no attempt was made to secure pure lines. The greatest growth among all the root tips was found in corn. In cases of excessive elongation fairly short and mostly unbranched secondary roots were formed. One individual root tip within a period of four and one-half months attained the astounding length of 1570 mm. with more than a hundred laterals (fig. 2). When such roots approached the end of their ability to continue elongation, lateral roots ceased forming with the result that several centimeters of the distal end were bare of secondaries (fig. 2). Occasionally, near the time when elongation ceased, there was a distinct thickening in the last 1 or 2 cm. of the tip. In cases where lateral roots developed profusely, the main axis failed to exhibit any great amount of elongation, a phenomenon which ROBBINS also observed. This behavior seems to support the view that the tip of the main axis, if vigorously growing, exercises an inhibiting influence upon the growth of the lateral branches of the axis. In general, lateral roots first appeared in corn no earlier than the seventh day and as late as the sixteenth day, with a few individuals producing no laterals at all. Such roots were thickened more than those possessing secondaries. Occasionally the cortex split away from the central cylinder near the proximal ends of the thickened roots, and also in a few of the roots with branches. Upon examination, the cortex of such roots was found to consist of huge cells elongated at right angles to the central cylinder. The interpretation is that the cortical parenchyma, when stimulated to hypertrophy, burst the bounds of the limiting epidermis, and in the resultant expansion split away from the central axis. This elongation and abnormal increase in size in the cells of the cortex was also observed in the

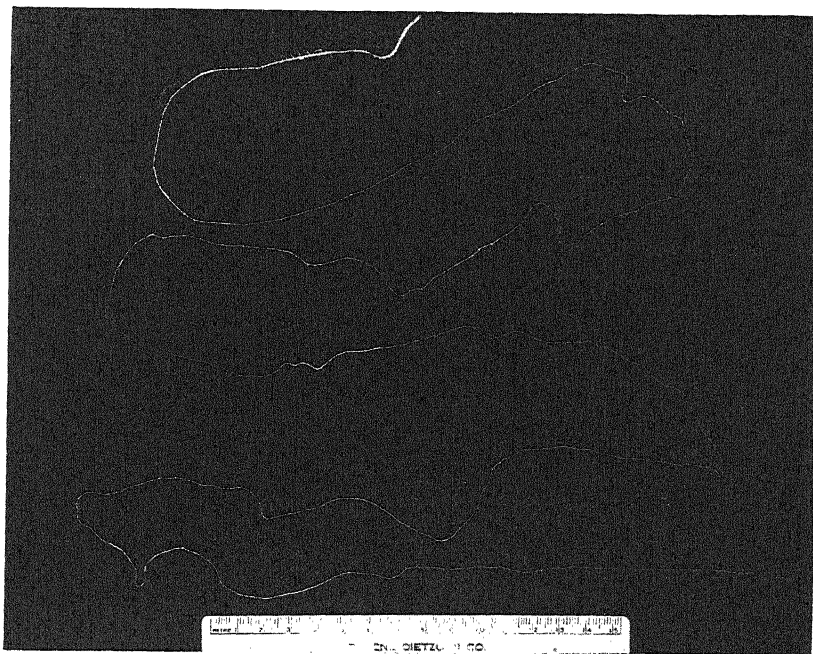


FIG. 2. Root of Reid's Yellow Dent corn, cut off when 10 mm. long, after four months of undisturbed growth in unchanged Pfeffer's solution plus glucose and peptone. This root, before drying, was 1570 mm. long and had 721 lateral roots.

swollen tips of the roots. In a few individuals, each lateral root was no more than 15 mm. long with the tip of each distinctly swollen. The cause of this interesting phenomenon is unknown, but as soon as the tips began to swell there was no further growth in length.

Hopi and sweet corn (fig. 1) behaved in a manner similar to that of dent corn with few exceptions. Neither attained lengths equal to those of dent corn; neither developed the characteristic swollen tips of dent corn; and the splitting of the cortex from the central cylinder was present to a lesser extent in both.

The root tips of *Gradus* pea grew slowly. After 7 to 10 days a pink coloration developed. When laterals appeared, they were always short, never exceeding 3 mm., and greatly thickened. In many cases these roots presented a corrugated appearance on their surfaces with the corrugations at right angles to the long axis of the root, resembling the contractile roots described by RIMBACH (6). Both cortex and epidermis grew faster than the central cylinder with the resultant wrinkling of the surface. This differs from the behavior of the dent corn roots, in which only the cortical cells

hypertrophied. There were some large cells in the cortex of the pea roots, but excessive cell division seemed also to have occurred.

With a few exceptions Burpee's Extra Early pea behaved in a manner similar to that of Gradus pea. They never elongated so much, were more slender, and developed secondary roots in only one instance.

Root tips of soybean after ceasing growth in the first week lay apparently inactive for 3 to 5 weeks, at which time a few hair-like secondaries appeared near the tip and in some instances grew 13 mm.

The uniformly abundant growth from the three varieties of corn suggested a short investigation to determine whether excised root tips from other monocots behaved equally well under the same conditions. Ten root tips each of wheat and onion in two series of five each were left to grow as near to maturity as possible. Onion was quite similar to Gradus pea except that the roots were more slender and developed no pigmentation. Wheat grew no more than 35 cm. and produced few secondaries. This seems to indicate that phyletic origin has no connection with the growth behavior of excised root tips.

### Summary

1. There was some correlation between growth behavior of excised root tips and the type of food stored in seeds.
2. All root tips from seeds or grains high in starch reserves were able to grow well.
3. The growth behavior of root tips from seeds selected for oil and sugar varied widely.
4. Root tips from seeds with high protein reserves were least able to maintain growth.
5. There were no diurnal rhythms of growth.
6. There was no correlation between growth behavior and phyletic origin of root tips.
7. Root tips from Reid's Yellow Dent corn, Hopi corn, sweet corn, and sunflower were able to maintain themselves in active growing condition for a longer time, and achieved a greater total elongation and final dry weight than those of any other species or variety studied.

JAMES MILLIKIN UNIVERSITY  
DECATUR, ILLINOIS

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# INTERMEDIATES OF VITAMIN B<sub>1</sub> AND THE GROWTH OF *TORULA*

WILLIAM J. ROBBINS AND FREDERICK KAVANAGH

(WITH SIX FIGURES)

In previous papers (2, 3, 4) from this laboratory the effect of vitamin B<sub>1</sub> or its intermediates on the growth of excised tomato roots and of *Phycomyces blakesleeanus* Burgeff has been reported. Excised tomato roots (2, 3) required for unlimited growth either vitamin B<sub>1</sub> or thiazole<sup>1</sup> in addition to mineral salts and cane sugar. Pyrimidine alone did not permit growth to occur. *Phycomyces blakesleeanus* (4), on the other hand, did not grow (3) unless supplied with vitamin B<sub>1</sub> or with both intermediates. We have been interested in determining whether there are organisms which require pyrimidine but not thiazole. SCHOPFER (5) has reported that *Rhodotorula flava* and *Rhodotorula rubra* are such organisms. We have examined eight species of *Torula* and have found some which require an external supply of both intermediates for good growth under the conditions of our experiments, some which require pyrimidine but not thiazole and some which require neither.

## Experimentation

A medium of the following composition was prepared:

KH <sub>2</sub> PO <sub>4</sub> .....	1.0	gm.
MgSO <sub>4</sub> · 7H <sub>2</sub> O .....	0.5	gm.
NH <sub>4</sub> NO <sub>3</sub> .....	0.05	gm.
Asparagine .....	0.5	gm.
Mineral supplements .....	0.1	cc. <sup>2</sup>
Dextrose (Cerelese) .....	50	gm.
Redistilled water .....	1000	cc.

The hydron concentration of this solution was adjusted to pH 5.5 by the addition of Na<sub>2</sub>HPO<sub>4</sub>. Twenty-five cc. of the above solution were placed in 125-cc. Erlenmeyer flasks of pyrex glass. The solutions were divided into four groups. No addition was made to those of group I.

<sup>1</sup> Where thiazole is referred to in this paper the 4-methyl-5-β-hydroxyethylthiazole is meant and where pyrimidine is mentioned we mean the 2-methyl-5-ethoxymethyl-6-aminopyrimidine. These compounds were used by WILLIAMS and CLINE (7) in synthesizing vitamin B<sub>1</sub> and are the intermediates in the formation of this vitamin.

<sup>2</sup> The mineral supplements were contained in a modification of HOAGLAND'S A to Z mixture prepared by adding to 18 liters of redistilled water: LiCl, 0.5 gm.; CuSO<sub>4</sub> · 5 H<sub>2</sub>O, 1.0 gm.; FeSO<sub>4</sub>, 1.0 gm.; H<sub>3</sub>BO<sub>3</sub>, 11.0 gm.; Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> · 18 H<sub>2</sub>O, 1.0 gm.; SnCl<sub>2</sub> · 2H<sub>2</sub>O, 0.5 gm.; MnSO<sub>4</sub> · 4 H<sub>2</sub>O, 7.0 gm.; NiCl<sub>2</sub> · 6 H<sub>2</sub>O, 1.0 gm.; Co(NO<sub>3</sub>)<sub>2</sub>, 1.0 gm.; TiOSO<sub>4</sub>, 1.8 gm.; KI, 0.5 gm.; NaBr, 0.5 gm.

To each flask of group II we added 30 units<sup>3</sup> (4.3  $\gamma$ ) of 4-methyl-5- $\beta$ -hydroxyethylthiazole, to each flask of group III 30 units (5.0  $\gamma$ ) of 2-methyl-5-ethoxymethyl-6-aminopyrimidine, and to each flask of group IV, 30 units of the thiazole and 30 units of the pyrimidine.

The flasks were sterilized at 12 lb. pressure for 20 minutes, and inoculated in triplicate with one drop of a suspension of *Torula*.

The *Torula* suspension was prepared by adding a loopful of *Torula* to 50 cc. of sterile distilled water. In removing the loopful of *Torula* care was taken to avoid including any of the wort agar on which the stock cultures were grown. The cultures were incubated at from 20° to 25° C.

The following *Torulæ* supplied through the courtesy of F. M. CLARK, Department of Bacteriology, University of Illinois, were used: *Torula hansen*, #2500; *T. sphaerica* (Hammar), #2504; *T. cremoris* (Hammar), #2512; *T. rosea*, #2519; *T. fermentati*, #2539; *T. kefyr*, #2540; *T. sanguinea* (Schimon), #2546; *T. laurentii*, #2547. The relative development of these eight organisms after ten days is shown in table I.

TABLE I

GROWTH OF VARIOUS SPECIES OF *Torula* IN A MEDIUM OF MINERAL SALTS, ASPARAGINE AND DEXTROSE WITH THE ADDITION OF THIAZOLE OR PYRIMIDINE AS INDICATED

ORGANISMS	NO ADDITION	THIAZOLE ONLY	PYRIMIDINE ONLY	THIAZOLE AND PYRIMIDINE
<i>Torula hansen</i> .....	++	++	++	++
<i>Torula sphaerica</i> .....	+	+	+	+
<i>Torula cremoris</i> .....	-	-	-	-
<i>Torula rosea</i> .....	-	-	++++	++++
<i>Torula fermentati</i> .....	+	+	+	++
<i>Torula kefyr</i> .....	-	-	-	-
<i>Torula sanguinea</i> .....	-	-	++++	++++
<i>Torula laurentii</i> .....	+	+	+	++++

- = little or no growth.

+ = light growth.

++ = medium growth.

+++ = fairly heavy growth.

++++ = very heavy growth.

The organisms did not respond alike. Two of them, *Torula cremoris* Hammar and *T. kefyr*, grew very little if at all in any of the solutions. Evidently none of the media used was suitable for the growth of these two species.<sup>4</sup> As a result no information on the significance of the thiazole or

<sup>3</sup> 1 unit is  $10^{-9}$  mole of the substance in question.

<sup>4</sup> A mineral nutrient solution containing asparagine and dextrose which was supplemented with vitamin B<sub>1</sub> and nicotinic acid amide gave negative results with these two organisms. They grew satisfactorily on a medium of agar, mineral salts, asparagine, dextrose, vitamin B<sub>1</sub>, and neopeptone.

of the pyrimidine in their development was secured. Two species, *T. hansen* and *T. sphaerica* Hammar, grew to the same extent in all four solutions as far as could be judged from the turbidity of the solutions. The growth of *T. hansen* was heavier than that of *T. sphaerica*. Either these two *Torulae* do not require thiazole or pyrimidine or they synthesize sufficient of these compounds from the constituents of the medium for maximum growth under the conditions used. On the basis of evidence presented later, we are inclined to believe the latter explanation to be the correct one.

The growth of *T. laurentii* (fig. 1) and of *T. fermentati* was markedly improved by the addition of the mixture of thiazole and pyrimidine but

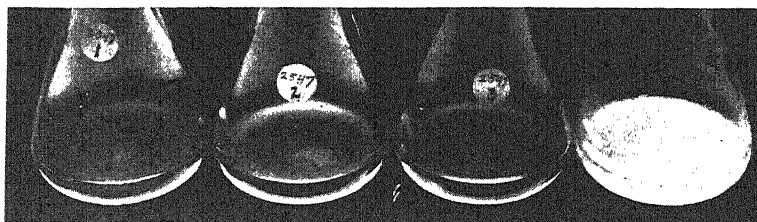


FIG. 1. *T. laurentii* grown in 1, basic medium (mineral salts, asparagine, and dextrose); 2, basic medium plus thiazole; 3, basic medium plus pyrimidine; 4, basic medium plus thiazole and pyrimidine.

little affected by either alone. The effect was greater for *T. laurentii* than for *T. fermentati*. These two species resemble *Phycomyces blakesleeana*s in requiring an external supply of both thiazole and pyrimidine, though the latter organism makes no growth in the absence of thiazole and pyrimidine while the two species of *Torula* under discussion grew slightly in the solution which lacked both thiazole and pyrimidine. It is possible that the small amount of growth of these two *Torulae* in the solutions lacking thiazole and pyrimidine or containing one of the compounds alone was made at the expense of small amounts of vitamin B<sub>1</sub> or of its intermediates carried in or with the cells of the original inoculum. Subcultures of these organisms from the growth in the absence of the intermediates to a solution of similar composition were not made. It would seem justifiable, however, to describe *T. laurentii* and *T. fermentati* as organisms requiring an external supply of pyrimidine and thiazole for good growth in the medium we have used. They may synthesize small amounts of the two intermediates, but if so the quantity formed under the conditions of our experiments was not sufficient for maximum growth.

*T. rosea* (fig. 2) and *T. sanguinea* grew very poorly in the solutions which lacked thiazole and pyrimidine and in those to which thiazole alone was added. They developed heavy growth in the solutions to which pyrim-



FIG. 2. *T. rosea* grown in 1, basic medium (mineral salts, asparagine, and dextrose); 2, plus thiazole; 3, plus pyrimidine; 4, plus thiazole and pyrimidine.

idine was added and in the solutions to which thiazole and pyrimidine had been added. Two possible explanations for these results may be offered. *T. rosea* and *T. sanguinea* may not synthesize either thiazole or pyrimidine and require only pyrimidine for normal growth, or they require both and synthesize the thiazole only. We are inclined to believe that the latter explanation is correct.

In order to substantiate in part the assumptions made on the ability of these various species of *Torula* to synthesize thiazole or pyrimidine, cultures of representatives of six species were sterilized at 12 lb. pressure for 10 minutes after 15 days' growth in the media described above. The sterile solutions containing the *Torula* cells and products of their metabolism

TABLE II

GROWTH OF *Phycomyces blakesleeanus* IN A MEDIUM OF MINERAL SALTS, ASPARAGINE AND DEXTROSE AND ADDITIONS GIVEN ABOVE IN WHICH VARIOUS SPECIES OF *Torula* HAD GROWN. COMPARE WITH TABLE I. FIGURES GIVEN ARE THE PH OF THE SOLUTIONS WHEN INOCULATED WITH THE *Phycomyces*

ORGANISMS	NO ADDITION	THIAZOLE ONLY	PYRIMIDINE ONLY	THIAZOLE AND PYRIMIDINE
<i>Torula hansen</i> .....	+++ 3.6	+++ 3.5	+++ 3.5	+++ 3.5
<i>Torula sphaerica</i> .....	-	+	-	+++
<i>Torula fermentati</i> .....	+	+	+	+++
<i>Torula laurentii</i> .....	- 3.6	-	-	+ 3.8
<i>Torula rosea</i> .....	- 4.3	- 4.2	++ 3.8	+++ 3.8
<i>Torula sanguinea</i> .....	- 3.7	- 3.7	+++ 3.6	+++ 3.9

+++ = Aerial mycelium 2 cm. high, sporangiophores and sporangia.

++ = Submersed mycelium with few scattered sporangiophores.

+ = Submersed mycelium covering bottom of flask; no aerial hyphae.

- = Little or no growth.

were inoculated with spores of *Phycomyces blakesleeanus*. This fungus requires both thiazole and pyrimidine for growth. Its development would therefore demonstrate the presence of both intermediates. Its failure to grow in a culture solution in which a species of *Torula* had grown might be the result of a deficiency of one or both of the intermediates or of the development of some harmful constituent in the solution.

The relative growth of *Phycomyces* in these solutions in which a species of *Torula* had grown is given in table II. Some determinations of the hydron concentrations of the solutions after the growth of the *Torula* and before inoculation with *Phycomyces* are also given in this table.

*Phycomyces blakesleeanus* developed fairly well in all four of the solutions in which *T. hansen* had grown. Aerial mycelium about 2 cm. high with sporangiophores and sporangia formed in all the culture solutions (fig. 3). The growth of the *Phycomyces* was probably limited by the con-

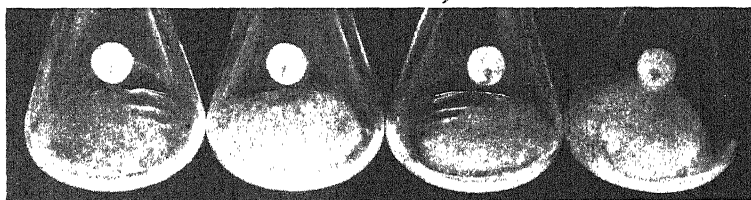


FIG. 3. *Phycomyces blakesleeanus* grown in solutions in which *T. hansen* had grown. From left to right: 1, basic medium; 2, plus thiazole; 3, plus pyrimidine; 4, plus thiazole and pyrimidine.

centration of asparagine (0.05 per cent.) in the original medium. We may conclude that *T. hansen* synthesized both thiazole and pyrimidine (or vitamin B<sub>1</sub>) from the constituents of the medium. From the growth of this *Torula* (see table I) in the four solutions we may conclude also that the amount of the thiazole and pyrimidine synthesized was adequate for maximum growth in the medium used. Otherwise some increase in growth

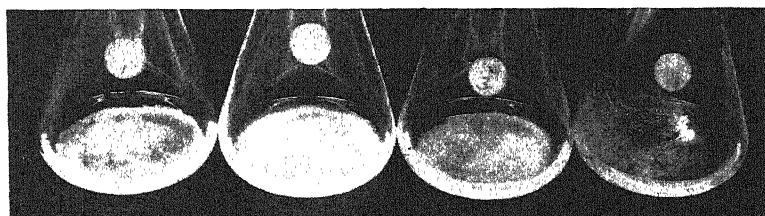


FIG. 4. *Phycomyces blakesleeanus* grown in solutions in which *T. fermentati* had grown. From left to right: 1, basic medium; 2, plus thiazole; 3, plus pyrimidine; 4, plus thiazole and pyrimidine.

would have occurred in the cultures to which thiazole and pyrimidine were added.

*Phycomyces* grew in all four of the solutions originally containing *T. fermentati*. The growth was greater in the solutions to which both thiazole and pyrimidine had been added (fig. 4). We conclude that *T. fermentati* synthesized both pyrimidine and thiazole but in smaller amounts per flask than *T. hansen* did. The amount of thiazole and pyrimidine synthesized was not sufficient for maximum growth of this *Torula* in the medium used (see table I) since the addition of the two intermediates improved its growth.

*Phycomyces* grew very little in the medium with no supplement or with pyrimidine in which *T. sphaerica* had grown but formed some submersed mycelium in the solution supplemented with thiazole (fig. 5). We conclude

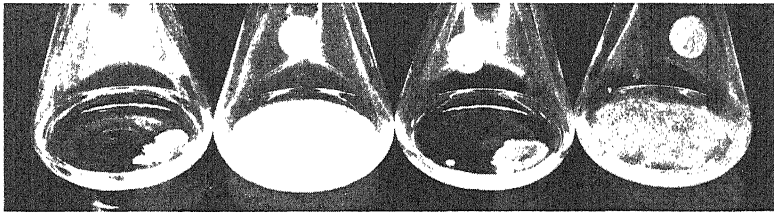


FIG. 5. *Phycomyces blakesleeenanus* grown in solutions in which *T. sphaerica* had grown. Left to right: 1, basic medium; 2, plus thiazole; 3, plus pyrimidine; 4, plus thiazole and pyrimidine.

that this *Torula* may have synthesized a small amount of pyrimidine but little or no thiazole. The failure of *Phycomyces* to develop more luxuriantly in these solutions would not appear to be the result of any injurious substances formed by the *Torula* since the growth of the *Phycomyces* was as great in the mixture of pyrimidine and thiazole as it was in the solutions in which *T. hansen* had grown. These results are not entirely clear when compared with the relative growth of *T. sphaerica* (see table I). If pyrim-

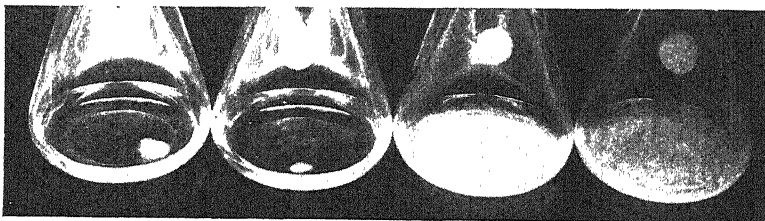


FIG. 6. *Phycomyces blakesleeenanus* grown in solutions in which *T. rosea* had grown. Left to right: 1, basic medium; 2, plus thiazole; 3, plus pyrimidine; 4, plus thiazole and pyrimidine.

idine and thiazole are required for growth by this *Torula*, we should have expected greater development of *Phycomyces* in the medium containing no supplements and in the medium with pyrimidine.

*Phycomyces* made little or no growth in the solutions with no supplements and those with thiazole only in which *T. rosea* or *T. sanguinea* had been inoculated (fig. 6). The growth in the solutions containing pyrimidine was better. We conclude that these two *Torulæ* synthesize little or no pyrimidine but form thiazole. The amount of thiazole per flask formed by the growth of *T. sanguinea* was somewhat greater than that formed by *T. rosea*. The relative growth of the two organisms in these solutions (table I) would suggest that the amount of thiazole formed by these two *Torulæ* was adequate for their maximum growth in the medium used.

*Phycomyces* grew little or none in the solution with no addition, the thiazole solution, and the pyrimidine solution in which *T. laurentii* had grown. Its development in the solution with both intermediates was poor. This suggests that this *Torula* had developed some material or materials which were injurious to *Phycomyces*. If this is correct our experiments would not permit us to draw conclusions on the formation of thiazole or pyrimidine by *T. laurentii*.

### Discussion

These observations on the growth of eight *Torulæ* in solutions lacking thiazole and pyrimidine and in solutions containing one or both of the intermediates, together with those on the subsequent growth of *Phycomyces blakesleeanus* in the same solutions, lend support to the hypothesis that vitamin B<sub>1</sub> is a fundamental growth factor. Some organisms make enough for adequate growth, others do not. Of those which do not, some synthesize neither the pyrimidine nor the thiazole in adequate quantities, others synthesize thiazole and some are capable of synthesizing pyrimidine. The result is that there are organisms which must be supplied with vitamin B<sub>1</sub> or both intermediates for satisfactory growth (*Phycomyces blakesleeanus*, *Torula laurentii*, *Torula fermentati*). There are organisms which must be supplied with pyrimidine or vitamin B<sub>1</sub> (*T. rosea*, *T. sanguinea*), and still others which must be supplied with thiazole or vitamin B<sub>1</sub> (tomato root).

It is possible to assume that the thiazole and the pyrimidine function in the organism as such, and that when vitamin B<sub>1</sub> is an effective growth substance it is split into its intermediates which then play their rôle in metabolism. As an alternate hypothesis we may assume that the vitamin molecule as such is essential and that the pyrimidine and thiazole synthesized by the organism or furnished from without are used to form the vitamin B<sub>1</sub> molecule. SCHOPFER favors the first of these possibilities. We

have presented evidence elsewhere supporting the second hypothesis. The demonstration that *Torulae* which grew in a solution lacking B<sub>1</sub> or its intermediates synthesized both intermediates supports our assumption. It is supported also by the demonstration that a *Torula* which grew in a medium supplemented with pyrimidine only synthesized thiazole.

OKUNUKI (1) noted the favorable action of red yeasts on the development of certain fungi. WASSINK (6) observed good development of *Phycomyces blakesleeanus* in a maltose medium which had become contaminated with a red yeast. Without the contamination little or no growth of the fungus occurred in the medium. These results may probably be explained by the formation of vitamin B<sub>1</sub> or of thiazole and pyrimidine by the yeasts concerned.

### Summary

Eight species of *Torula* were grown in a medium of mineral salts, asparagine, and dextrose, and the same medium to which the thiazole alone, the pyrimidine alone, or both intermediates of vitamin B<sub>1</sub> were added. Two species failed to grow in any of the four media; the growth of two was unaffected by the supplements; two grew distinctly better when both intermediates were used as supplements but were unaffected by either intermediate alone; the growth of two was much increased by the addition of pyrimidine or of pyrimidine and thiazole but unaffected by thiazole alone. By cultivating *Phycomyces blakesleeanus* in the solutions in which various species of *Torula* had grown it was demonstrated that a *Torula* which grew in the basic solution had synthesized thiazole and pyrimidine and one which grew in the solution supplemented with pyrimidine alone had synthesized thiazole.

NEW YORK BOTANICAL GARDEN

AND

UNIVERSITY OF MISSOURI

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# RELATIONSHIP OF THE ORGANIC ACIDS OF TOBACCO TO THE INORGANIC BASIC CONSTITUENTS<sup>1</sup>

GEORGE W. PUCHER, HUBERT BRADFORD VICKERY,  
AND ALFRED J. WAKEMAN

## Introduction

There is little doubt that one of the important functions of the organic acids commonly found in relatively substantial proportions in leaf tissues is to provide a means whereby the hydrogen-ion activity of the solution in the cells is maintained within the proper limits for the particular species. There is considerable literature on the buffer capacity of plant juices (3, 4, 5), and it is obvious that the combined effects of the partially neutralized organic acids, and of the phosphoric acid, as well as of the amino acids, and possibly, to some extent, of the soluble proteins result in systems that are remarkably stable with respect to change in hydrogen-ion activity.

Owing to the inadequacies of the methods available until recently, few reliable studies have been made of the detailed organic acid composition of leaves, and for determinations of total acidity many investigators have depended entirely upon titration of water extracts of the tissues. Such data furnish a measure merely of that part of the water-soluble acids that remains unneutralized at the reaction of the cells and give little indication of the total quantity present either as soluble or insoluble salts. In order to ascertain the true organic acidity it is necessary to liberate the acids from combination and isolate them as a group; the total quantity present can then be determined by titration between properly selected pH limits. With this information available it becomes possible to investigate the relationships between the chief acid- and base-binding substances of the tissue.

Data obtained in the course of a study of these factors in tobacco leaf tissue are described in the present communication. We were fortunate in having at our disposal a series of samples derived from plants that had been grown with especially careful control of the inorganic constituents of the fertilizer applied. Complete analyses of the ash of these samples had been made by Dr. E. M. BAILEY of the Connecticut Agricultural Experiment Station and published in connection with BAILEY and ANDERSON'S studies (1) of the effect of various soil treatments on the technical quality of the leaf. We are indebted to them for permission to carry out organic acid analyses of these samples and to recalculate and quote their data.

<sup>1</sup> The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

### Experimentation

The samples used in the experiments consisted of leaves that had been subjected to the processes of curing and of fermentation. It was therefore desirable first to establish the nature of the effect of these operations on the organic acids. Samples of tobacco leaves picked the same day were allowed to cure for various periods in the customary way. Each sample consisted of 60 leaves of approximately the same total initial weight. The results of the analyses were calculated upon an arbitrary basis of 1 kilo of original fresh weight of each individual sample. The data referring to the nitrogenous constituents and the carbohydrates have been discussed in a previous publication (7). The results of the determinations of the total organic acidity are given in table I.

TABLE I

TOTAL ORGANIC ACIDITY OF TOBACCO LEAVES AT VARIOUS STAGES IN THE CURING PROCESS

HOURS	ACIDITY*
	<i>mcq./kgm.</i>
0	320
41	367
64	340
87	285
111	320
159	272
185	275
207	347
231	357
279	308
303	339
Average	320.9 $\pm$ 22.1

\* Data are expressed in milliequivalents per kilogram of original fresh weight of each sample.

These figures show that little or no change occurs in the total organic acidity throughout the period of curing. The probable error is  $\pm 6.9$  per cent., which is the order of precision to be expected for measurements of this type. Alterations in the relative proportions of individual acids may, and in fact do, occur, but there is no substantial conversion of organic acids into nonacidic metabolic products, nor is there evidence for the accumulation of acid end products of the metabolism of other substances.

The information available on the effect of fermentation on the organic acids is very limited. HALEY, NASSET, and OLSON (2) carried out determinations of the ether-soluble organic acidity of parallel samples of cured and fermented tobacco, employing a method not unlike that used in this

laboratory. The average of nine determinations made on web tissue before and after fermentation differed by only 3 per cent., and it seems fair to assume therefore that such changes as may occur during fermentation are of a minor nature. This conclusion is supported by inferences drawn from the reaction of the tissues. The reaction of water extracts of our fermented samples ranged from pH 6 to 6.5—values of exactly the same order of magnitude as those of extracts from many samples of cured tissue. Important alterations in the proportion of organic acids present would have changed this reaction appreciably inasmuch as the inorganic basic constituents did not change. For the present purposes, however, comparisons are to be made between samples, all of which had been subjected to fermentation in the same way; minor changes that may have taken place in the organic acids accordingly have no significance.

The samples were derived from two different crops, 1924 and 1927, respectively a dry and a rainy season, and represented a considerable variety of cultural treatments. The two crops differed widely in the technical quality of "fire-holding capacity," the 1927 crop being classified as good, the 1924 crop as poor in this relation. The soil treatments of each individual plot were the same in each year, and had been maintained the same for the crops grown during the intervening years on the same plots. All samples were graded by experts, and the so-called darks, "D," and seconds, "S" were employed for the present work. These two grades represent leaves derived respectively from the upper and from the lower parts of the plant.

The detailed analyses of the ash and of the intact tissue were recalculated for the present purpose in terms of milliequivalents per 100 gm. of dry leaf. The positive ions calcium, magnesium, potassium, nicotine, and ammonium, and the negative ions phosphate, chloride, sulphate, and nitrate, were employed. Phosphorus and nicotine were reckoned as monovalent ions in the calculations because the tissue reactions were close to pH 6. The analytical figures for sulphate reported by BAILEY and ANDERSON represent both organic and inorganic sulphur, but a study of the sulphur distribution of the samples showed that approximately 70 per cent. was in the form of inorganic sulphate; much of the balance doubtless represents sulphur combined in proteins. The factor 0.7 was therefore used to correct the total sulphur determinations of BAILEY and ANDERSON in order to arrive at an approximation to the quantity of inorganic sulphate. An analogous correction was not applied to the phosphate data although the determinations represent total phosphorus in the ash, and a part of this may have originated from organic phosphorus compounds. It is assumed that all of the phosphorus represents monovalent phosphoric acid radicals as there is little doubt from the nature of the known organic phosphorus compounds of plant tissues that a part of the phosphorus even in such compounds fulfills

this function. In any case, the error involved in this assumption has little effect upon the conclusions to be drawn as phosphoric acid forms so small a part of the total inorganic acidity present. The nature of the compounds of silicon in the tissues presents a very puzzling problem, and it would appear from the present data that very little if any of the silicon dioxide determined in the ash represents silicic acid or analogous acidic substances.

The sum of the positive ions present in the ash, together with the ammonia and nicotine, may be taken to represent the total acid-binding capacity of the tissues. The sum of the inorganic negative ions represents the base-binding capacity of the tissues due to inorganic acids. In all cases there is a large excess of positive ions, and it is the purpose of the present paper to show that this excess is closely related to the quantity of ether-soluble organic acids in the intact tissue.

It should, perhaps, be emphasized that the comparison is made between the excess of positive ions present at approximately the tissue reaction of pH 6 and the *whole* of the ether-soluble organic acids that can be titrated between the limits pH 7.8 and 2.6. This involves the assumption that the proportion of organic acids in combination with bases is substantially fixed, notwithstanding possible variation from sample to sample in the chemical composition of this group of substances. Furthermore, in the calculation of the bases it is assumed that the inorganic constituents of the ash are all present in the intact tissue in ionic form capable of salt formation. This is certainly not entirely true; for example, a part of the magnesium is involved in the fresh tissues in complex combination in the form of chlorophyll. The fate of this part of the magnesium after the technical processes of curing and fermentation have been carried out is entirely unknown.

The data assembled in table II permit a comparison between the excess acid-binding capacity of the individual samples from the 1927 crop and the total organic acidity as determined by the method of PUCHER, VICKERY, and WAKEMAN (6) after ether extraction.

The degree with which one variable depends on the variation of another is probably most easily appreciated from a consideration of the correlation coefficient calculated in the customary way. In the present case we have two sets of data, one obtained from plants fertilized with various potassium compounds, the other with lime. Each set of 12 samples contains 6 lots of leaves graded as "darks" and 6 graded as "seconds." These grades imply a difference in position on the plant as well as intangible elements of technical "quality," but for the present purpose this may be neglected and the two series grouped together.

The correlation coefficient between the excess positive ions and the total organic acidity for the 12 samples from plants grown on the potash plots is  $0.833 \pm 0.060$ ; that for the other set is  $0.905 \pm 0.035$ . A similar calcula-

tion for the entire set of data from the 1924 crop (13 samples) gave a correlation coefficient of  $0.812 \pm 0.067$ . These coefficients indicate that the quantities are closely dependent on each other.<sup>2</sup>

Although the statistical analysis of the data leads to the conclusion that the two variables are closely interdependent, it in no way suggests which is the independent and which the dependent variable. It is not possible to conclude that certain plants absorbed more basic ions from the soil and therefore elaborated more organic acids. It may be equally true that certain plants manufactured more organic acids and therefore absorbed more inorganic base from the soil. Some entirely different factor may indeed have been the true independent variable, and it is quite possible that the mutually related organic acidity and excess basic ion content varied in response to the changes in this other factor.

Inspection of the data of table II shows that the inorganic acidic components of the tissue (negative ions) play a minor rôle in maintaining the balance between the positive and negative ions of the tissue. The average of the total inorganic anions amounts to only about one-sixth of the average total cations in the samples from the potash plots and to one-tenth for the samples fertilized with lime. Organic acidic substances, particularly in the latter case, obviously dominate the situation.

It must be emphasized that we are in a position to deal from the analytical point of view only with that part of the organic acids of the tissues that can be extracted with ether under suitable conditions. No accurate estimate can at present be made of base-binding substances that cannot be extracted with this solvent. The tobacco leaf contains considerable quantities of pectins and allied compounds, some of which possess base-binding properties. If the assumptions upon which the present calculations have been founded are correct, it would appear that roughly one-third of the excess positive ions are, on the average, combined with acidic substances other than ether-soluble organic acids.

The acids actually determined by the method employed are chiefly malic, oxalic, and citric; these three make up about four-fifths of the ether-soluble acidity. The exact chemical nature of this group is, however, immaterial to the present discussion.

If it be accepted that the high correlation between the excess positive ions and ether-soluble organic acidity in tobacco leaf tissues demonstrates a mutual interdependence between these quantities, inferences may be drawn from average values and extended to the consideration of other sets of

<sup>2</sup> The high significance of these coefficients may be appreciated from comparison with FISHER's table (SNEDECOR, G. W. Statistical methods. Ames, 1937, p. 125). For 10 degrees of freedom the coefficient at the 1 per cent. level of significance is 0.708, for 11 degrees of freedom it is 0.684.

TABLE II

RELATIONSHIP BETWEEN POSITIVE AND NEGATIVE IONS OF ASH, TOGETHER WITH AMMONIA AND NICOTINE, AND TOTAL ORGANIC ACIDITY OF INTACT TISSUE IN SAMPLES OF FERMENTED TOBACCO LEAF FROM CROP OF 1927

POTASH SERIES*						LIME SERIES*					
SAMPLE NO.	GRADE	TOTAL POSITIVE IONS	TOTAL NEGATIVE IONS	EXCESS POSITIVE IONS	TOTAL ORGANIC ACIDITY	SAMPLE NO.	GRADE	TOTAL POSITIVE IONS	TOTAL NEGATIVE IONS	EXCESS POSITIVE IONS	TOTAL ORGANIC ACIDITY
608	D	385.8	81.4	304	175	620	D	430.4	34.9	396	245
610	D	402.0	65.2	337	207	622	D	396.4	65.9	331	223
612	D	385.7	64.3	321	177	624	D	413.1	39.8	373	272
614	D	403.7	63.7	340	176	626	D	388.3	44.4	344	216
616	D	430.4	84.9	346	205	628	D	450.8	27.7	423	304
618	D	407.4	70.8	337	206	630	D	388.3	40.1	348	212
Average		402.6	71.7	331	191			411.2	42.1	369	245
609	S	443.0	98.0	345	183	621	S	470.2	33.3	437	302
611	S	445.7	87.7	358	250	623	S	411.1	50.5	361	250
613	S	424.6	73.6	351	219	625	S	444.8	29.0	416	290
615	S	418.1	57.9	360	213	627	S	383.7	47.6	336	253
617	S	434.6	69.6	365	262	629	S	488.3	27.0	461	335
619	S	435.7	53.3	382	211	631	S	404.1	23.7	380	272
Average		433.6	73.3	360	223			433.7	35.2	398	284
Correlation coefficient r = 0.833 ± 0.060						Correlation coefficient r = 0.905 ± 0.035					

\* Figures are milliequivalents per 100 gm. of dry tissue.



samples. It may be useful to present the data from this point of view. In table III are shown the average values of the organic acidity and of the excess positive ions of samples of grade D from two crops fertilized each in two ways. In the upper portion of the table are details from which the relative order of magnitude of the individual bases and of the inorganic acidity may be appreciated. Calcium and potassium clearly occupy the dominant position among the bases.

The organic acidity of the samples grown on the potash plots in 1924 was distinctly higher than that of the samples grown in 1927. In the later crop, there was a marked decrease in total positive ions, partly due to a diminution in calcium content, and there was a corresponding decrease in inorganic acidic ions. The compensation was not, however, complete so that the ratio between the excess positive ions and the organic acidity changed from 1.52 to 1.73.

The samples from the lime-fertilized plots showed a slight increase in organic acidity and in excess positive ions. There was a marked decrease in calcium and potassium in the 1927 crop which was only partially compensated by an increase in magnesium. The substantial drop in inorganic negative ions, however, completed the compensation and preserved the balance between the excess positive ions and the organic acids. The ratio did not change significantly.

If the potash series of 1924 is compared with the lime series of the same

TABLE III

RELATIONSHIP BETWEEN POSITIVE AND NEGATIVE IONS OF SAMPLES OF TOBACCO OF GRADE D  
DERIVED FROM THE CROPS OF 1924 AND 1927

	POTASH SERIES*		LIME SERIES*	
	1924	1927	1924	1927
Calcium .....	180	148	200	161
Potassium .....	166	173	150	128
Magnesium .....	67.7	55.6	54.7	90
Nicotine .....	14.6	10.6	16.3	13.7
Ammonia .....	33.2	15.6	39.3	18.6
Total positive ions .....	462	403	460	411
Total negative ions (inorganic acidity) .....	107	71.7	110	42
Excess positive ions .....	355	331	350	369
Organic acidity .....	234	191	236	245
Ratio $\frac{\text{excess positive ions}}{\text{organic acidity}}$ .....	1.52	1.73	1.48	1.51

\* Figures are averages of from four to six individual samples and are expressed in milliequivalents per 100 gm. of dry tissue.

year, it is clear that there was no significant change in the organic acidity nor in the excess positive ions. The inorganic acidity is also practically the same, the slight difference in excess positive ions being attributable to minor differences in calcium and potassium. But if the potash series of 1927 is compared with the lime series of that year, there is an important increase in organic acidity in the samples from the lime plots. There is only a small increase in total positive ions, but there is a substantial diminution in the inorganic negative ions, so that the excess of positive ions increases materially. Nevertheless, the change falls a little short of being strictly proportional to the change in organic acids, the ratios being 1.73 and 1.51 respectively.

A similar comparison between the "D" samples and the "S" samples from the 1927 crop is shown in table IV. These grades to a considerable extent represent position of the leaves on the plant, the "S" grade being mainly bottom leaves, the "D" grade, upper leaves. The bottom leaves from the potash plots contain more organic acids than top leaves from plants fertilized in the same way. The inorganic negative ions are practically the same, and it is clear that the balance of ions is maintained by the organic acids. The same is true of the two sets of samples from the lime plots. Differences between the lime and potash samples within each group are mostly caused by the lower inorganic negative ions in the lime series with correspondingly higher organic acids.

TABLE IV

RELATIONSHIP BETWEEN POSITIVE AND NEGATIVE IONS OF SAMPLES OF GRADE D AND GRADE S FROM THE CROP OF 1927

	"D" SAMPLES* TOP LEAVES		"S" SAMPLES* BOTTOM LEAVES	
	POTASH SERIES	LIME SERIES	POTASH SERIES	LIME SERIES
Calcium .....	148	161	177	180
Potassium .....	173	128	181	120
Magnesium .....	55.6	90	62.7	112
Nicotine .....	10.6	13.7	5.7	9.9
Ammonia .....	15.6	18.6	7.4	11.5
Total positive ions .....	403	411	434	433
Total negative ions (inorganic acidity) .....	71.7	42	73.3	35.2
Excess positive ions .....	331	369	360	398
Organic acidity .....	191	245	223	284
Ratio $\frac{\text{excess positive ions}}{\text{organic acidity}}$ .....	1.73	1.51	1.61	1.40

\* Figures are averages of six samples and are expressed in milliequivalents per 100 gm. of dry tissue.

It is apparent that the ether-soluble organic acidity of tobacco leaf tissue may vary between relatively wide limits, and that this variation is associated with the manner in which the plants have been fertilized. The data indicate with some clearness that the fluctuations are in the direction which, from the chemical composition of the tissue, may be expected to maintain an equilibrium between the positive and negative ions such that the hydrogen-ion activity of the tissues does not vary in any significant manner. Many factors undoubtedly come into play, but the nature of the inorganic ions that are assimilated and the total amount present have a direct influence. The organic acids are therefore definitely concerned in the problem of inorganic nutrition, and a comprehensive study of the manner in which these substances may vary in response to the cultural treatment of the plant is much to be desired.

### Summary

1. If it be assumed that the major constituents of the ash of cured and fermented tobacco leaves, with the exception of silicon dioxide, represent respectively basic or acidic ions combined essentially as salts in the cells, and if, in addition, allowance is made for the nicotine, ammonium, and nitric acid of the tissues, it is possible to calculate the total positive ions and the total negative ions present in terms of chemical equivalents.

2. In all cases examined there is a large excess of positive ions and the quantity so found is closely correlated with the quantity of ether-soluble organic acids as determined by a suitable titration method.

3. The organic acids occupy a dominating position with respect to the balance of positive and negative ions in the tissues, and it is inferred that these substances are closely concerned in the phenomena of inorganic nutrition.

CONNECTICUT AGRICULTURAL EXPERIMENT STATION  
NEW HAVEN, CONNECTICUT

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# GROWTH OF CABBAGE SEEDLINGS IN SAND CULTURE, AS AFFECTED BY DELAYED APPLICATION OF NUTRIENT SALTS

A. A. DUNLAP

(WITH TWO FIGURES)

## Introduction

When some kinds of seeds are thickly planted, the resulting seedlings are likely to develop excessively elongated hypocotyls and to be otherwise unsuitable for transplanting. It has long been known that excessive elongation of many kinds of stems, including hypocotyls, is generally correlated with inadequate light intensity, as in partial or complete etiolation, when conditions of temperature and water supply are adequate and when inorganic nutrients are available. Without referring particularly to numerous earlier papers on the etiolation of ordinary plants, reference may be made to POPP's (6) observation that stem elongation in early growth stages of soy bean was approximately inversely proportional to the intensity of the light employed. Reference may be made also to the experiments of VAN OVERBEEK (5), who found that the rate of hypocotyl elongation and the final length attained by radish hypocotyls were only about half as great when the seedlings were grown under ordinary conditions as when they were grown in darkness. That different kinds of plants should show great differences in respect to this influence of light is only to be expected (MACDOUGAL's classic book on light effects, 4), and the physiological problem thus raised is surely one of great complexity. In connection with the study here reported, the employment of artificial illumination in the greenhouse at night was only slightly effective in retarding and limiting hypocotyl elongation in cucumber seedlings.

In this study it has been found that under ordinary greenhouse conditions seedlings of cabbage, cucumber, and tomato generally become "drawn," showing some of the growth symptoms of etiolation, when the seeds are sown too close together, but exhibiting a quite satisfactory degree of stockiness when they are allowed to grow a few centimeters apart. Dense stands of perfectly satisfactory seedlings have been grown in an ordinary greenhouse, however, by employing sand as substrate, to which suitable nutrient salts had been added in suitable concentrations (2). Ammonium nitrate was found to be very satisfactory when initially added at the rate of about 0.5 gm. per liter of sand, the pH value of the solution used being between 5 and 7; the sand used contained a trace of available phosphorus. Some other ammonium salts gave similar results, but leaf injury was likely to follow their use unless

a potassium salt (such as  $\text{KCl}$ ,  $\text{K}_2\text{SO}_4$ ) was also added initially, at suitable concentration. In any event, this desired stunting effect of an  $\text{NH}_4^+$  salt was secured only when the initial salt concentration was properly adjusted. With concentration too high, toxic effects were produced; with concentration too low, the crowded seedlings showed the usual condition of partial etiolation. Injury from the use of  $\text{NH}_4^+$  salts was especially likely to occur when tall sand columns were employed with automatic tray irrigation (3).

The present paper reports some recent greenhouse experiments in which satisfactorily stocky seedlings were obtained in crowded stands without the use of  $\text{NH}_4^+$ . These were grown in pots of washed sand with tray subirrigation (3) and without addition of any nutrient salt to the sand until 10 days after the seedlings had emerged. At that time a delayed fertilizer treatment, with  $\text{KNO}_3$  and  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ , was applied. The experiments were carried out at the Connecticut Agricultural Experiment Station in the late spring and early summer of 1937.

### Experiments

A fairly coarse, reddish-brown sand with angular grains, from a deep pit near New Haven, was used as culture medium. This was first washed in hot ( $65^\circ \text{C}$ .) tap water, to remove most of the silt and soluble materials and to insure against damping-off (2). The wet sand was placed in glazed butter crocks each having a diameter of 17 cm., a depth of 10.5 cm., and a capacity of about 2500 ml. Every crock had a 5-mm. hole in the bottom to allow for constant-level subirrigation of the type described by DUNLAP and LIVINGSTON (3). The air-dry weight of the contents of each crock was approximately 3.7 kg. The crocks stood in tap water the surface of which was about 1 cm. above the bottom of the sand mass, which held about 750 ml. of water when in equilibrium with the free water below. For a series of cultures with seedlings of cabbage (*Brassica oleracea* L.), which will now be described, there were 9 crocks.

After the sand had been leveled off about 1.5 cm. below the crock rim, 3 of the 9 crocks (group B) each received 100 ml. of a 2-salt nutrient solution containing 0.01 gm. mol of  $\text{KNO}_3$  and 0.001 gm. mol of  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ . This solution had been found to produce as good growth of seedlings under these conditions as was obtained with more complex solutions. To the remaining 6 crocks no nutrient solution was added at the start. Three of them (group A) received no fertilizer at any time, but the remaining 3 (group C) subsequently received the addition just mentioned, 10 days after the seedlings had emerged.

Next, 200 seeds (germinative capacity about 80 per cent.) were planted in each of the 9 crocks and covered with about 0.5 cm. of washed sand, the surface being then sprinkled with water. The crocks stood about 8 cm.

apart on a watertight concrete bench with raised edge. This constituted the subirrigation tray, in which water was maintained at a depth of 1 to 2 cm. Thus the cultures were well subirrigated at all times. No artificial shade was used and on clear days, except for a few hours in early morning and late afternoon, the cultures were in direct sunlight.

In all cultures the seedlings emerged from the sand about 4 days after sowing. Ten days after their emergence most of those without fertilizer treatment (groups *A* and *C*) had only one very small true leaf, in addition to the cotyledons. The cotyledons were about 21 mm. above the sand, and the average fresh weight of aerial parts was about 8 gm. per 100 seedlings. At the same time, each of the seedlings of the initially fertilized cultures (group *B*) had a small true leaf; the cotyledons were about 50 mm. above the sand, and the average fresh weight of the aerial parts was about 25 gm. per 100.

Nutrient solution was at this time applied to 3 of the 6 previously unfertilized cultures (group *C*), as already mentioned, by sprinkling it over the seedlings and washing it into the sand by means of a light sprinkling of water. About 3 days later the seedlings that received this delayed application of fertilizer showed greatly accelerated growth and rapidly became darker green. They continued to grow vigorously until the cultures were discontinued on the 24th day after emergence. At that time it appeared that all cultures had reached a stage where any considerable further growth could not occur without further additional application of nutrient salts. Meanwhile, the seedlings that had received fertilizer treatment at the start, which

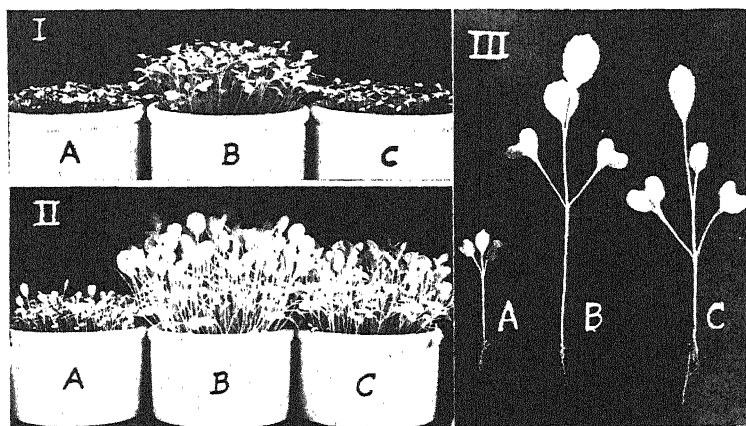


FIG. 1. Cabbage seedlings in sand cultures; I, 10 days after emergence; II, 20 days after emergence; III, representative seedlings 20 days after emergence. *A*, no nutrient salts applied at any time; *B*,  $\text{KNO}_3$  and  $\text{Ca}(\text{H}_2\text{PO}_4)_2$  added to sand when seeds were sown; *C*, the same salts added to sand 10 days after emergence of seedlings.

were already much advanced on the 10th day after emergence, continued to grow, but those without any fertilizer treatment grew only slightly, remaining very small—about as they were on the 10th day. The general appearance of the cultures on the 10th and 20th days after emergence and representative seedlings for the 20th day after emergence are shown in figure 1. Some growth data secured on the 10th and the 24th day after emergence are set forth by the diagrams of figure 2. For each time of observation these diagrams show the following features: Average height (millimeters) of cotyledons above sand; average length (millimeters) of stem above coty-

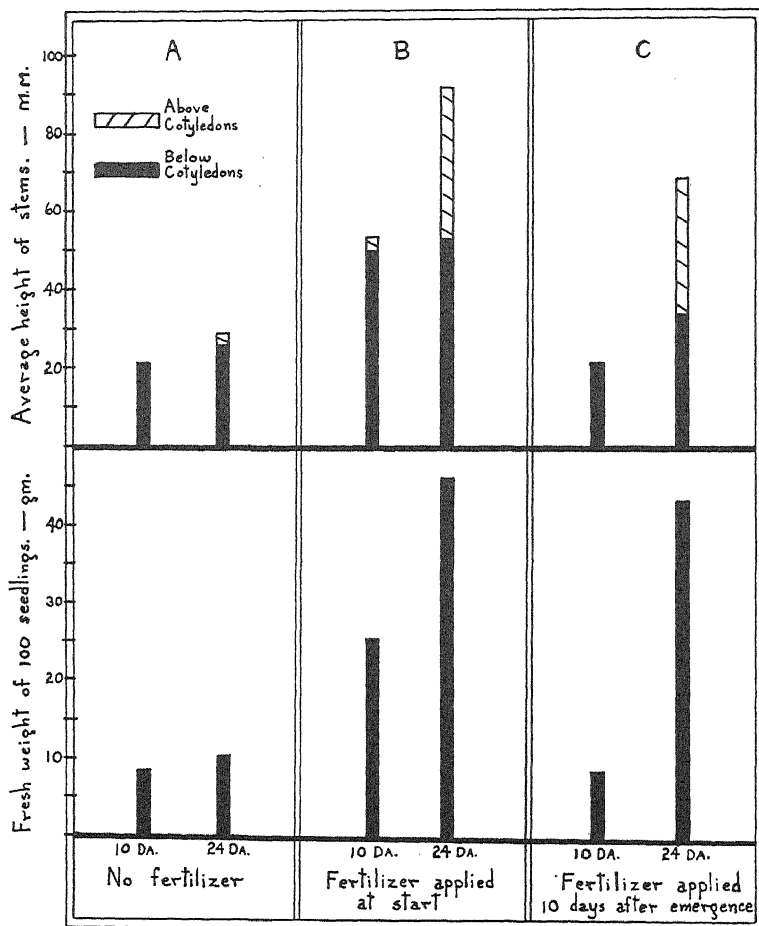


FIG. 2. Growth diagrams for seedlings represented in figure 1, showing length of stems and fresh weights on the 10th and 24th days after emergence of seedlings. Height of stem above cotyledons plus height of stem below cotyledons is total height of stem above sand.



ledons; average total height of stem—*i.e.*, average height (mm.) of terminal stem growing point above sand; and average fresh weight (gm.) of aerial portions per 100 seedlings.

At the end of the experimental period the seedlings showed marked differences according to the fertilizer treatments they had received. Those grown without any fertilizer (*A*) were very small indeed, with only a single leaf; those with initial fertilization (*B*) were apparently well grown but much "drawn" (as though somewhat etiolated); and those with delayed fertilization (*C*) were greatly superior—much stockier and shorter. Although both were grown in very dense stands, the seedlings of the *B* group were not well suited for transplanting into soil but those of the *C* group were in very excellent condition for transplanting.

Further examination of the figures furnishes a more detailed picture of these growth differences and the manner in which they were brought about.

In the first 10 days after emergence the height of the cotyledons above the sand became about 21 mm. in the absence of added nutrient salts (*A*) but it became about 50 mm. when the salts were present from the start (*B*). This height was subsequently increased from about 50 mm. only to about 53 mm. for the initially fertilized seedlings (*B*), and for those with delayed fertilization (*C*) this increase was greater; namely, from about 21 to about 33 mm. At the end of the experimental period the hypocotyls were very much shorter in the cultures with delayed fertilization (*C*) than in those with initial fertilization (*B*); indeed, with delayed fertilization the cotyledon height above the sand was at that time only about two-thirds as great as it had been with initial fertilization on the 10th day. It is thus clear that the final marked superiority and stockier appearance of the seedlings produced with delayed fertilization was in part due to this difference in hypocotyl elongation and final length.

The elongation of the stem above the cotyledons is also of interest. On the 10th day after emergence the average length of the entire epicotyl was only about 3 mm. with initial fertilization (*B*), and it was too short to measure when no fertilizer had been applied. In the following 14 days the length of the epicotyl increased from about 3 to about 39 mm. with initial fertilization, and from about zero to about 35 mm. with delayed fertilization. It thus came about that the total stem height above the sand was finally only about 68 mm. with delayed fertilization, while it was about 92 mm. with initial fertilization.

Although the seedlings with delayed fertilization were finally much shorter than those with initial fertilization, the final average fresh weight per 100 seedlings was almost as great with delayed fertilizer treatment (44 gm.) as with initial treatment (46 gm.). It is interesting to note that the fresh-weight value on the 10th day was only about 8 gm. for the *C* seedlings

while it was about 25 gm. for the *B* seedlings, and that these values increased during the next 14 days at different rates, attaining about the same magnitude on the 24th day. More than one-half of the final fresh weight of the initially fertilized seedlings represents growth during the first 10 days after emergence, but only about one-fifth of the final weight of the seedlings with delayed treatment represents growth before the nutrient salts were applied.

In summarizing, it may be said that delayed treatment was superior to initial treatment in that, although final fresh weight was about the same for both, final length of hypocotyl was much shorter and the length of the epicotyl was somewhat shorter with delayed treatment. Results similar to those just presented were sometimes obtained when the nutrient salts were applied 7 or 8 days after emergence of the seedlings from the sand, but when application was delayed only 3 or 4 days these effects were not produced. With longer periods, such as 20 days after emergence, more pronounced effects were obtained than with the 10-day period, and greater foliar development was noted on the seedlings with delayed fertilization.

Experiments similar to these were carried out with cucumber (*Cucumis sativus* L.) and tomato (*Lycopersicum esculentum* Mill.) and these plants were essentially like cabbage in their response to delayed application of nutrient salts.

### Discussion

A physiological explanation of the results described above would surely involve consideration of growth-promoting substances and the relations of their actions to mineral nutrition and perhaps to light intensity and temperature of both air and soil. Under the conditions of the experiments with cabbage it appears that, in the absence of added salts (*A*), hypocotyl elongation practically ceased before the 10th day, but that when it ceased these organs still retained for a time their capacity to elongate further if salts were then supplied. An increment of about 12 mm. was added to the average hypocotyl length after the delayed application of nutrient salts on the 10th day, and that increment amounted to more than 36 per cent. of the final height of the cotyledons above the sand in the *C* cultures. When the added salts were present from the start (*B*), that increment was only about one-fourth as great (about 3 mm.), amounting to only about 6 per cent. of the final height of the cotyledons. It might be supposed that it was inadequacy of  $\text{NO}_3^-$  supply to hypocotyl and cotyledons which brought the period of hypocotyl elongation to a close somewhat before the 10th day in the *A* cultures, but it is difficult to imagine that hypocotyl elongation ceased for that reason in the *B* cultures, where the supply of that important ion was obviously still adequate for vigorous epicotyl growth throughout the succeeding 14 days.

Whatever conditions may have brought about the cessation of hypocotyl

elongation, it is clear that the average rate of that process in the first 10 days after emergence—and presumably throughout its whole grand period—was very much more rapid when the added salts were present than when they were not. This difference in rate may readily be supposed to have been caused by an accelerating influence exerted by the added salts, presumably by  $\text{KNO}_3$  or by  $\text{NO}_3^-$ . That influence may have been effective directly on the elongating cells of the hypocotyl itself or indirectly through the cotyledons, or in both of these ways. VAN OVERBEEK (5) has recently reported that, when the cotyledons of radish seedlings (*Raphanus sativus* L., which is rather closely related to *Brassica*) were removed early, hypocotyl elongation was at first very slow, becoming more rapid and reaching its completion only after the epicotyls had attained considerable growth. He concluded that hypocotyl elongation could not occur in the usual way without the presence of a growth hormone, which appeared to be derived from the cotyledons or, in their absence, from the developing epicotyl. On the basis of that conclusion it may be supposed that the presumably salt-starved cabbage cotyledons of the present study were less effective to furnish the hypocotyls with requisite hormone than were the cotyledons of the initially fertilized cultures. Thus both the duration and the rate of hypocotyl elongation in the seedlings without initial fertilization may have been limited not only by inadequacy of  $\text{NO}_3^-$  supply but also by inadequacy of hormone supply. Furthermore, this supposed partial failure of the salt-starved cotyledons to deliver hormone to their hypocotyls may itself have been associated with  $\text{NO}_3^-$  deficiency in their own tissues, for AVERY, BURKHOLDER, and CREIGHTON (1) found that stem tips of sunflower and tobacco contained little or no growth hormone when inadequately supplied with  $\text{NO}_3^-$ , while the hormone content of these tissues was much greater when the supply of  $\text{NO}_3^-$  had been plentiful.

Elongation of the epicotyls began after hypocotyl elongation had practically ceased in the cultures without any fertilization, and it began somewhat earlier with the other treatments. Without added salts it began some time after the 10th day but the cultures with initial fertilization showed epicotyls about 3 mm. long on that day. In the succeeding 14 days the unfertilized epicotyls elongated very little (about 3 mm.) but those with initial and with delayed fertilization elongated rapidly, the rates being about equal. The final epicotyl length was somewhat greater with initial fertilization (about 39 mm.) than with delayed fertilization (about 35 mm.). The growing epicotyls probably produced their own growth hormones (5), but it does not appear that their rate of elongation was related in any clear way to the concomitant elongation of the hypocotyls, which had nearly completed their elongation before epicotyl elongation began. It is remarkable that epicotyl elongation in the final 14-day period was a little more rapid with initial fertilization than when the salts were added only at the beginning of that

period; although the initial supply of added nutrient salts had probably become more or less depleted by the 10th day, the residue was obviously sufficient for vigorous epicotyl development in the following 14 days, as has been noted. As far as the final stockiness of the seedlings with delayed fertilization was related to shortness of epicotyls, it was clearly due to earlier cessation of epicotyl elongation rather than to retardation of that process.

Of course the growth of these seedlings involved other features than those of hypocotyl and epicotyl elongation, notably, extensive development of the root system and foliar development and expansion. The superiority of the cultures with delayed fertilization was clearly ascribable, in considerable part, to their greater uniformity in size and more advanced leaf growth. The information at hand concerning these peculiarities is insufficient, however, to warrant any attempt to interpret them in terms of salt nutrition, hormones, etc. Nevertheless, the data presented in figure 2 concerning fresh weight of aerial parts (including hypocotyls, cotyledons, epicotyl stems, and leaves) may be examined briefly. Although the final average fresh-weight values derived from initial and from delayed fertilizer treatment are essentially alike (about 46 and 44 gm. per 100 seedlings), the rates of weight increase were very different. With initial fertilization the average fresh weight increased in the 14-day period from about 25 to about 46 gm., the increment being only about 21 gm., but when the salts were applied on the 10th day the corresponding increase was from about 8 to about 44 gm., the increment being about 36 gm. Of the final fresh weight, in the former case about 46 per cent., and in the latter about 82 per cent., had been produced in the last 14 days of growth. The seedlings produced by the method of delayed fertilization were therefore superior to those grown with initial fertilization in that a much greater proportion of their tissues had resulted from recent rapid development.

The results of the present study seem to lead to the suggestion that the stockier seedlings produced with the 10-day delay in fertilization might perhaps have possessed their superior characteristics to a still greater degree if they had been allowed to remain without fertilization somewhat more than the 10 days after emergence. Since it is reasonable to expect seedlings of different species or varieties to respond more or less differently to this technique of delayed fertilization, it seems likely that the delay requisite for most satisfactory results may be longer for some forms and shorter for others. A tentative rule may be proposed; to wit, that fertilizer salts are to be withheld for a while after hypocotyl elongation has ceased, until the hypocotyls have lost their physiological capacity to elongate further even after the salts are added. Also, other environmental features, besides fertilization and the time of its application, may be worthy of consideration as this technique is developed further; perhaps, for example, a suitably maintained low tem-

perature—of the sand or of both sand and air—throughout the preliminary period of salt starvation might give results even more satisfactory than those described in this paper. From the standpoint of practical horticulture as well as from that of plant physiology, this procedure of delayed fertilization with tray subirrigation seems highly promising and worthy of further experimental study.

### Summary

1. This paper presents results of greenhouse experiments with cabbage seedlings closely grown in glazed crocks of washed sand with constant-level tray subirrigation. Some of the crocks were initially supplied with fertilizer salts ( $\text{KNO}_3$  and  $\text{Ca}(\text{H}_2\text{PO}_4)_2$ ); others received the same fertilizer treatment only on the 10th day after the seedlings had emerged from the substrate; and still other crocks received no fertilizer at any time. The experiments were discontinued on the 24th day after seedling emergence and the seedlings finally obtained differed markedly according to their fertilizer treatment.

2. Seedlings grown without any fertilization were obviously salt-starved, greatly stunted, with only a very small true leaf. Those that had received initial fertilization were well developed but they appeared as though partially etiolated, having long, slender hypocotyls and epicotyls; they were unsuited for transplanting. Those that had received delayed fertilization were like those with initial fertilization in fresh weight of aerial parts but each had relatively shorter hypocotyls and epicotyls. These seedlings with delayed fertilization were remarkably stocky and well suited for transplanting.

3. Physiological reasons for the pronounced superiority of the cultures with delayed fertilization are briefly considered, especially with reference to hypocotyl elongation, epicotyl development, and fresh weight of the aerial parts.

4. The procedure of delayed fertilization with washed sand and with tray irrigation is highly promising from the horticultural point of view, for it seems to offer, at least under greenhouse conditions similar to those of New Haven in spring and summer, a very simple and almost automatic means for quantity production of seedlings suitable for transplanting. This general procedure should be useful also in scientific studies on the relations of seedling development to environmental conditions and to hormone influences.

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TEXAS AGRICULTURAL EXPERIMENT STATION  
COLLEGE STATION, TEXAS

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# ISOLATION OF A HEXOSEMONOPHOSPHATE FROM PEA LEAVES

W. Z. HASSID

## Introduction

HARDEN and YOUNG (7, 8) showed that phosphates accelerate the fermentation of sugars by yeast-juice and discovered hexosediphosphoric acid in the products resulting from such fermentation. Since that time the relation of phosphates to carbohydrate metabolism has been the subject of intensive study. The investigations of the several hexosephosphates isolated from yeast and muscle contributed much toward the knowledge of the complex carbohydrate metabolism of the animal body and served particularly to elucidate the chemistry of muscle.

In recent years data have been accumulated which indicate that hexosephosphates are also formed in the higher plants and that they play perhaps as important a part in the carbohydrate metabolism of the plant as they do in the animal body.

The fact that phosphates stimulate the respiratory sugar metabolism in higher plants suggests that the formation of phosphoric esters may occur as part of this process (4, 10). It is also known that when preparations of germinating peas, beans, barley, etc., are allowed to act upon the hexosediphosphoric acid of HARDEN and YOUNG, products are formed which are characteristic of sugar breakdown in yeast or muscle (1, 14, 15). When phosphoglyceric acid is used as a substrate, pyruvic acid is formed (16).

Evidence that phosphorylation takes place in the plant was first presented by BODNÁR (5). He demonstrated that inorganic phosphorus disappeared when added to ground peas in the presence of toluene. BARRENSCHEEN and ALBERS (2) found that the acid-soluble phosphorus increased in irradiated *Elodea canadensis* and during germination of rye. Later BARRENSCHEEN and PANY (3) showed the existence of hexosephosphates in *Elodea* after it was kept for a certain irradiation period in a dilute sugar solution to which inorganic phosphorus was added.

TÁNKÓ (23), using pea preparations to which inorganic phosphorus was added, isolated a mixture of hexosediphosphate and hexosemonophosphate similar to the HARDEN and YOUNG and the ROBISON esters. In addition; some esters were present which differed in constitution from the known phosphoric esters. BURKARD and NEUBERG (6) have shown that both glucose and fructose monophosphate are formed in beet leaves.

In the present work the writer also isolated a hexosephosphate from pea leaves, which is, apparently, a mixture of the glucose- and fructose-phosphoric esters. This affords more evidence to support the view that phos-

phorylation takes place in the higher plants with the production of phosphoric esters similar to those found in yeast or muscle, and also suggests a possible parallelism between carbohydrate metabolism in the higher plants and in yeast or muscle.

### Experimentation

#### ISOLATION OF THE HEXOSEMONOPHOSPHATE

The plant material was prepared and the hexosephosphate isolated according to a slightly modified procedure described by BURKARD and NEUBERG (6).

Peas (variety Giant Stride) were planted on May 25, 1937, and harvested in July at about the time of the appearance of pods. Lots of 3 kg. of fresh leaves were quickly thrown into containers with 10 liters of boiling 95 per cent. alcohol, boiled for 15 minutes and allowed to stand overnight. The dark alcoholic solution was decanted, the leaves washed on a Büchner funnel with alcohol, dried at 28° C., and ground to a powder.

Two hundred and fifty gm. of the ground plant material were mixed with 5 liters of 0.4 N hydrochloric acid and stirred for 1½ hours. The solution was filtered off, cooled to 5° and neutralized with dilute sodium hydroxide to a weakly acidic reaction of pH 5 and then cooled again to the same temperature. One hundred and sixty cc. of 10 per cent. solution of copper sulphate and 500 cc. of calcium hydroxide were added and stirred for ten minutes. The precipitate was quickly removed by filtration, washed, and ground. It was then dissolved with dilute sulphuric acid, the acid being added until the solution gave a faintly blue color with congo red. During this operation the solution was kept cool by the addition of cracked ice to the mixture. The calcium sulphate formed was separated by filtration, the filtrate treated with hydrogen sulphide, the copper sulphide removed by filtration, and the hydrogen sulphide removed from the solution by a stream of air. To the liquid, which was kept ice-cooled, a warm saturated solution of barium hydroxide was added until it gave a red color to phenolphthalein; then carbon dioxide was introduced until the color disappeared. A 50 per cent. barium acetate solution was slowly added until no further precipitation occurred. The precipitate was filtered off and the liquid evaporated to about 100 cc. under reduced pressure at 40°, the solution filtered, and precipitated with basic lead acetate. The precipitate was washed on a Büchner funnel and then suspended in water. A stream of hydrogen sulphide was introduced into the solution, while the mixture was continuously shaken. The filtrate was cleared from the hydrogen sulphide by a stream of air, then barium hydroxide was added to the red end-point of phenolphthalein, after which it was filtered, and the solution precipitated with neutral lead acetate. This precipitate was filtered off and reserved for later treatment.



Basic lead acetate was added to the filtrate, and the precipitate, after being washed, was decomposed with hydrogen sulphide as before. The hydrogen-sulphide-free filtrate was neutralized again with barium hydroxide, and after filtration was added drop by drop to five times its volume of alcohol. The barium salt formed was filtered off on a sintered glass crucible, washed with alcohol, and dried in a desiccator. The precipitate previously obtained by the addition of neutral lead acetate was similarly treated.

About 0.8 gm. of the crude barium salt was obtained from two 250-gm. lots of the dried plant material.

This crude barium salt was dissolved in alcohol, 20 cc. of water was added, and the mixture shaken. The insoluble precipitate which separated out was filtered off, and the filtrate was treated with neutral lead acetate until no more cloudiness appeared. The precipitate was filtered off and the solution was treated with basic lead acetate. The lead salt formed was washed with water and changed into the barium salt in the manner described. It was precipitated with alcohol, dissolved in water, and precipitated again with alcohol. The yield of the barium hexosephosphate, after drying in vacuum at 70°, was 0.35 gm.

#### ANALYSIS OF THE HEXOSEPHOSPHATE

The barium salt of the hexosephosphoric acid ester was not obtained in the absolutely pure state.

<sup>1</sup> Found .....	C, 17.95 per cent.; P, 7.15 per cent.; Ba, 36.6 per cent.
Calculated for $C_6H_{10}O_5PO_4Ba$ .....	C, 18.22 per cent.; P, 7.85 per cent.; Ba, 34.73 per cent.

The ratio of the Ba to P is therefore 1.155. Since only a limited amount of the material was available, further purification, according to the method of ROBISON (21), by conversion into the brucine salt and regeneration back into the barium salt was not attempted.

BURKARD and NEUBERG's ratio, Ba:P, for the barium hexosephosphate isolated from beet leaves was 1.336 (6). After regeneration from the brucine salt they obtained a Ba:P ratio of 1.063.

The specific rotation of the barium salt was determined by dissolving 10 mg. of the substance in a 2.5 cc. volumetric flask and polarizing in a 100-mm. (1 cc. capacity) tube. The reading was  $-0.3^\circ$ . The specific rotation of the salt  $[\alpha]_D$  was therefore of the order of magnitude of  $-25^\circ$ .

The aldose value, determined iodimetrically according to the method of MACLEOD and ROBISON (12), was 11.8 per cent. and with the ferrieyanide method (9) 38.0 per cent. The Seliwanoff reaction carried out on the substance as described by ROE (22) was positive. These values indicate that the carbohydrate fraction of the compound is a mixture of about 30 per cent. glucose and 70 per cent. fructose.

<sup>1</sup> The analyses were carried out semi-microchemically.

The negative rotation of the compound suggests that the fructose component might be the fructose-1-phosphate described by TÁNKÓ and ROBISON (24), as this is the only barium hexosephosphate with a negative rotation thus far reported.

An osazone was prepared from the barium salt as follows: 0.15 gm. of the salt was dissolved in 2 cc. of water, and the exact amount of dilute sulphuric acid necessary to precipitate the barium was added. The filtrate was treated in a test tube with 0.2 gm. of phenylhydrazine hydrochloride and 0.3 gm. of sodium acetate, and then heated on a water bath for half an hour. An osazone separated out on cooling. When recrystallized from a mixture of chloroform and alcohol, according to NEUBERG and REINFURTH (20), its melting point was 150°, which corresponds with values given in the literature.

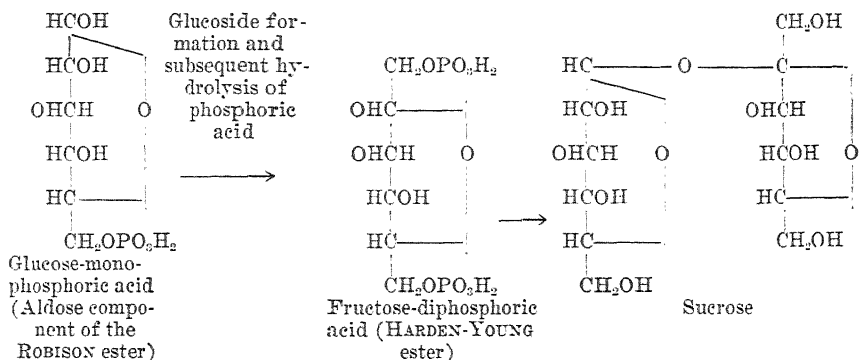
### Discussion

NEUBERG and LEIBOWITZ (17, 18, 19) showed that when the proper enzyme acts upon either glucose or fructose ester an equilibrium mixture of glucose- and fructose-phosphoric acid ester results. The ease with which these two esters are formed, coupled with the fact that glucose- and fructose-phosphoric acid ester exist in actively metabolizing leaves of peas and sugar beets (6), which produce sucrose abundantly, suggests that phosphorylation of the hexose sugars is a necessary step for the synthesis of sucrose. Furthermore, the chemical structure of sucrose, containing the furanose (unstable or  $\gamma$  form) form of fructose and the stable pyranose form of glucose in its molecule, can be adequately explained only by assuming that phosphorylation of these two monosaccharides takes place before the synthesis of the sucrose molecule.

The fructosediphosphoric as well as the fructosemonophosphoric acid ester are known to possess the furanose structure. MORGAN (13) showed that the same methylhexosediphosphoric acids are obtained whether the methylation is carried out at room temperature or at 60°, the latter condition being favorable to the formation of methylfructopyranose, containing the stable 2:6 or amylene oxide ring. MORGAN and ROBISON concluded from these facts that the second phosphoric acid group is attached to the terminal C atom, thereby preventing the formation of the stable 2:6 oxide ring. LEVENE and RAYMOND (11) arrived at a similar conclusion from the results of methylation experiments. Phosphorylation of the fructose molecule evidently takes place when it is in the straight chain form. Subsequently, when the ring is formed, it results in a furanose structure because the hydroxyl of the sixth carbon atom is occupied in the phosphoric acid ester linkage, thus preventing the formation of the pyranose structure.

Now, if we assume a direct glucosidal linkage between this five-mem-

bered ring of phosphorylated fructose and the six-membered glucose-mono-phosphoric acid and then subsequent hydrolysis of the phosphoric acid groups, the molecule of sucrose would result:



### Summary

1. A hexose monophosphate was isolated from pea leaves, which proved to be a mixture of glucose- and fructose-phosphoric acid esters. The negative rotation of the barium salt of this hexosephosphoric acid seems to indicate that the fructose component is, probably, fructose-1-phosphate.
2. It is suggested that phosphorylation of the glucose and fructose components is a necessary step in the synthesis of sucrose in the plant.
3. The occurrence of the unstable furanose form of fructose in the sucrose molecule is explained by assuming that phosphorylation of the fructose and glucose takes place prior to the synthesis of sucrose in the plant.

The writer is indebted to Professor D. R. HOAGLAND for his encouragement of this work and helpful suggestions, and to Mr. L. W. TUTTLE, who did the major portion of the analytical work.

UNIVERSITY OF CALIFORNIA  
BERKELEY, CALIFORNIA

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# INFLUENCE OF OSMOTIC PRESSURE ON SPORULATION BY *BACILLUS SUBTILIS*

JAMES L. ROBERTS, WELDON C. WHITE AND  
ELIZABETH OJERHOLM

(WITH TWO FIGURES)

Studies on the physiology of bacterial endospore formation have in many cases involved the addition of one or more compounds to a basal medium, and a determination of the effect of these upon the percentage of cells appearing as spores. The consequences, so far as the spore crop is concerned, of the addition of electrolytes (FITZ-GERALD, 5; FABIAN and BRYAN, 3), fermentable carbohydrates (ESTY, 2; DE SMIDT, 7), vapors of fat solvents (MICHAJLOWSKY, 6), antiseptics, and many other compounds have been studied by this method.

Among the bacteria, forms can be found which are more resistant to great changes in osmotic pressure than are any other forms of life (FALK, 4). Many species of bacteria, however, in common with other plants and animals, are readily affected by changes in the osmotic pressure of their menstruum (CURRAN, 1). It is well known that certain physical properties of a medium may influence the degree of endospore formation occurring within that medium. According to our best knowledge, little or no work has been done to determine the influence of osmotic pressure on sporulation. Studies involving the addition of materials of small molecular size could be more easily interpreted if there were available more definite knowledge of the effect of osmotic pressure on spore formation. The present work was undertaken to determine whether osmotic pressure is a factor sufficiently important to warrant consideration in future studies of bacterial sporulation.

## Methods

A 0.5 per cent. Bacto-peptone water was used as a basal medium to which the various materials used in raising the osmotic pressure were added. The basal solution was prepared in one large vessel and distributed in 25-cc. volumes into 6-oz. prescription bottles with screw caps. The following materials were then weighed or measured into the bottles of basal solution:

1. Ten concentrations of KCl (0.05 M to 0.5 M)
2. Ten concentrations of NaCl (0.05 M to 0.5 M)
3. Ten concentrations of  $\text{MgSO}_4$
4. Ten concentrations of  $\text{MgCl}_2$  (0.033 M to 0.333 M)
5. Ten concentrations of  $\text{CaCl}_2$  (0.033 M to 0.333 M)
6. Ten concentrations of sodium acetate (0.05 M to 0.5 M)
7. Ten concentrations of glycerol (0.1 M to 1 M)

8. Six concentrations of lactose (0.1 M to 0.6 M)
9. Ten concentrations of agar (0.01 to 0.1 per cent.)
10. Ten concentrations of NaCl (0.05 M to 0.5 M)  
in basal medium with 0.1 per cent. agar

The media were sterilized in 15 pounds of steam pressure for 30 minutes and the hydrogen-ion concentrations of the various solutions were then ascertained to be between pH 6.4 and 6.6. Inoculation was accomplished by two drops of a 24-hour peptone water culture of the "K strain" of *Bacillus subtilis* obtained from the culture collection of the University of Texas. The cultures were incubated in a horizontal position with the bottle caps loosened. The incubation temperature used was 37° C. Under these conditions there was little or no pellicle formation.

Smears were prepared from each of the cultures after two and four days of incubation. It was intended that the final pH and osmotic pressure values should be determined at the completion of four days of incubation, but time did not permit a determination of these until the eighth day.

The smears were steamed in 5 per cent. aqueous malachite green, de-stained with distilled water, and counterstained with 5 per cent. mercurochrome. Approximately 500 cells were counted on each smear, and from these the percentage of spores was calculated.

In general, growth was not visibly inhibited by the osmotic pressures of the solutions we have used in these studies. Osmotic pressures greater than 23 atm. have not been investigated since only rarely do media contain sufficient osmotically active materials to produce greater pressures.

## Results

As would be expected, the pH of most of the media became increasingly alkaline to 7.5 to 8.0 during eight days of growth. Those media containing glycerol and lactose were exceptions in that they became slightly more acid than the uninoculated control media. Because the results of spore counts in media containing glycerol and lactose may not be comparable with the results obtained in the remainder of the test solutions, they are omitted in future considerations. The percentage of sporulation in the presence of each of these two materials was lower than with the other chemicals tested.

Growth of *B. subtilis* seemed to cause a slight increase in the osmotic pressure of the menstruum after eight days of incubation. Since this change was always less than one atmosphere, it is considered negligible.

The curves representing sporulation in any series of media after two and four days of incubation (fig. 1) are sufficiently similar to indicate no excessively great experimental error in the mechanics of the determinations. The gravest danger is in drawing conclusions when so few compounds have been studied, and when it has been impossible to completely isolate the effects



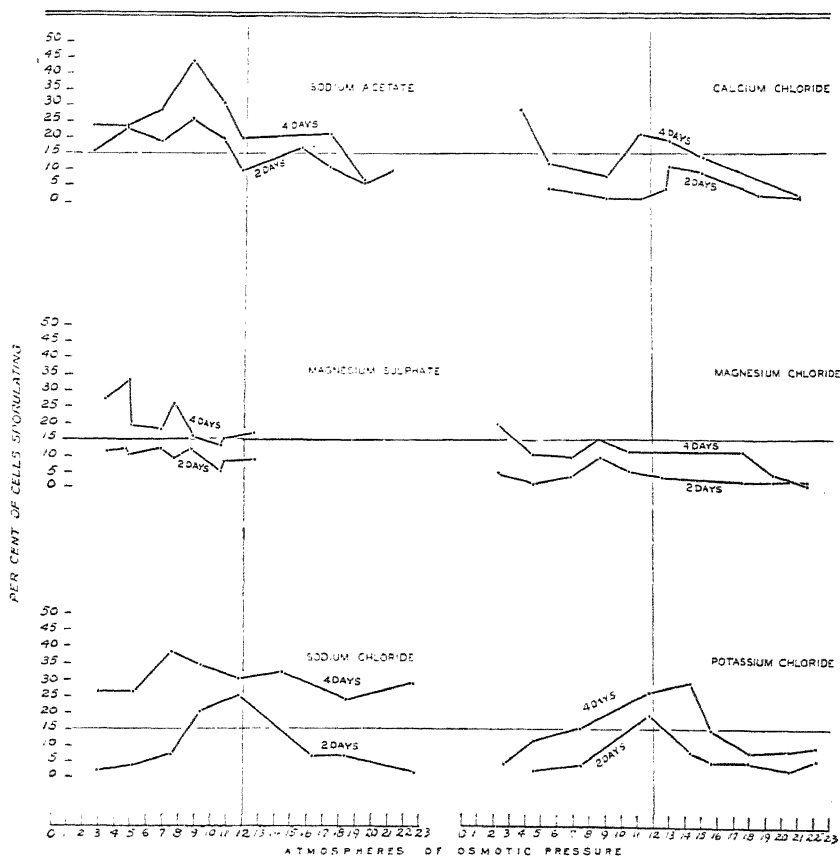


FIG. 1. Sporulation by *Bacillus subtilis* in peptone broth as influenced by variously induced osmotic pressures.

of the osmotic pressure from the specific effects of the added chemicals. Specific influences of the chemicals are evident in the results presented in figure 1. It will be noted, however, that the various curves have some characteristics in common. In view of this, it seemed both fair and helpful to compound the results from all the media plotted in figure 1. The resulting histogram is shown in figure 2.

The percentages of sporulating cells in media containing agar are much higher than in other media. Therefore, the results with agar are of little value for comparison and are omitted from figures 1 and 2. The osmotic pressure of media containing agar has little or no effect upon sporulation.

The influence of osmotic pressure, at least in the case of *B. subtilis*, seems not to be sufficiently great to warrant serious attention in future spore studies. As shown in figure 2, the percentages of sporulation in media with osmotic pressures ranging from 2 to 18 atm. are not significantly different.

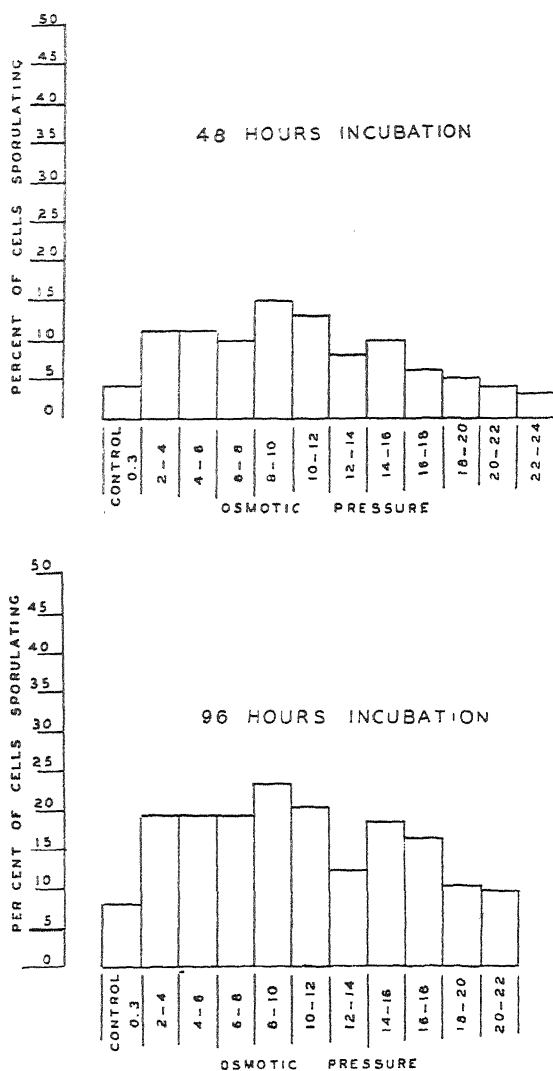


FIG. 2. Composite results (excepting those from media containing agar) showing effect of osmotic pressure on sporulation by *B. subtilis*.

There does seem to be a slight inhibition of sporulation by pressures above 18 atmospheres and below 2 atmospheres.

DEPARTMENT OF AGRICULTURAL BACTERIOLOGY  
 THE UNIVERSITY OF WISCONSIN  
 DEPARTMENT OF BOTANY AND BACTERIOLOGY  
 THE UNIVERSITY OF TEXAS

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## BRIEF PAPERS

### INFLUENCE OF THE LENGTH OF DAY ON THE DORMANCY OF TREE SEEDLINGS

FELIX G. GUSTAFSON

(WITH ONE FIGURE)

Since the introduction of the term "photoperiodism" by GARNER and ALLARD (2), 18 years ago, almost all of the work on the subject has dealt with reproductive responses of plants to length of day, and only very little with vegetative responses. In fact, until recently it was generally considered that photoperiodism had reference only to sexual responses. In 1923 (3) GARNER and ALLARD, among their other experiments, made some preliminary observations on the retention of leaves by *Rhus glabra* and *R. copallina* when kept under long days. They found that these plants retained their leaves longer under long days than under short days, but eventually shed them even under long day conditions. They also noted that *Liriodendron tulipifera* plants grown in the greenhouse produced new leaves earlier under long day conditions than under short day conditions. BOGDANOV (1) working near Leningrad shortened the photoperiod of 1- and 2-year-old seedlings of several tree species and found that this treatment shortened their seasonal growth period. MOSHKOV (7), also working near Leningrad, found the same to be true for the plants he studied. He also found that, though a shorter than normal photoperiod shortened the seasonal period of growth, the total amount of growth made by the plant per growing season was not always decreased. Thus *Pyrus ussuriensis* and *Salix babylonica* grew more under 10, 12, and 14 hours of daylight than under the normal 20-hour day. There were also morphological changes brought about by changing the photoperiod. The early cessation of growth under the shorter than normal photoperiod enabled the wood to ripen and the plants were more frost resistant than plants grown under normal length of day.

From his work with several species of trees, KRAMER (5) came to the conclusion: "variation in length of day may, to a considerable extent, control the duration of the growing season of certain tree species." In a later paper KRAMER (6) describes a situation in which a street light caused an increased growth period in shrubs; this continued growth prevented the wood from maturing, and the stems were winter killed.

Several years ago the writer became interested in the vegetative response of tree seedlings to length of day. At that time some 3-year-old transplants of *Picea canadensis* and *Pinus resinosa* were brought into the laboratory early in the fall, and potted for class use. After the class was through with

the plants, they were kept in the greenhouse for possible use in the spring semester. A relatively short time after the plants were brought into the greenhouse the white spruce plants began to grow and made an elongation of several centimeters, but the pines showed no sign of growth activity. Sometime in February or March a few of the dormant pine seedlings were placed under 500-watt lamps and given a photoperiod of about 16 hours. Within a few weeks these plants commenced to grow, but the plants receiving no additional light remained dormant. In fact, the plants given the normal day length did not grow in length during all of the following summer. As only a few plants were used, no conclusions could be drawn from the experiments and no further observations were made for 5 years.

Early in the fall of 1935 one hundred 3-year-old *Pinus resinosa* plants similar to the ones mentioned above were brought into the greenhouse and potted in 4-inch pots. These plants remained in a low-temperature greenhouse during the winter of 1935-1936. The following summer the plants were repotted into 6-inch pots and placed back in the same greenhouse, but they made no growth in length during this period, and many died. Early in the fall of 1936 twenty-five of these plants were put into a cold frame. They were left there until March 5, 1937, when they, together with the remaining plants that had not been exposed to the freezing temperature were placed in a warm greenhouse. Of the hundred plants that were potted in 1935 there were only 39 good plants left; of these 14 had been exposed to a freezing temperature, and 25 had remained at a temperature of from 10° to 12° C. From the latter group 14 plants were selected, and, together with the 14 plants that had been allowed to freeze, were placed in a part of the greenhouse where they received only the natural length of day. The remaining 11 plants of the 25 were placed under 500-watt lamps and were given a photoperiod of about 16 hours. The temperature was the same for the two lots of plants.

On May 21, the plants were examined. The buds were counted on each plant and those that had opened were also counted. On the non-frozen plants under the short day conditions, 29.5 per cent. of all the buds showed a development of one or more needles. In most of the buds only a few needles were produced and there was no elongation of the stem. Only 6 buds out of a total of 261 grew as much as 1 cm. On the corresponding plants given the long days, 87.5 per cent. of all of the buds showed some growth. Every apical bud, and on most plants at least two other buds, grew as much as one centimeter in length. The plants that had been exposed to the outdoor temperature until March 5 and normal greenhouse conditions with short days since that time had 87.6 per cent. of all buds open. This is the same as for the plants under the light. Every apical bud developed and in most plants 2 other buds showed considerable growth. The total

growth in length was somewhat greater than that made by the plants under the lamps.

Both lots of plants receiving a normal photoperiod became dormant after whatever growth they had made during the first 2 months in the warm greenhouse, and they remained dormant until the middle of the summer when they were discarded. On the other hand, the plants exposed to the 16-hour photoperiod produced a number of buds and many of these made a second growth during the early summer (fig. 1, plant 3).

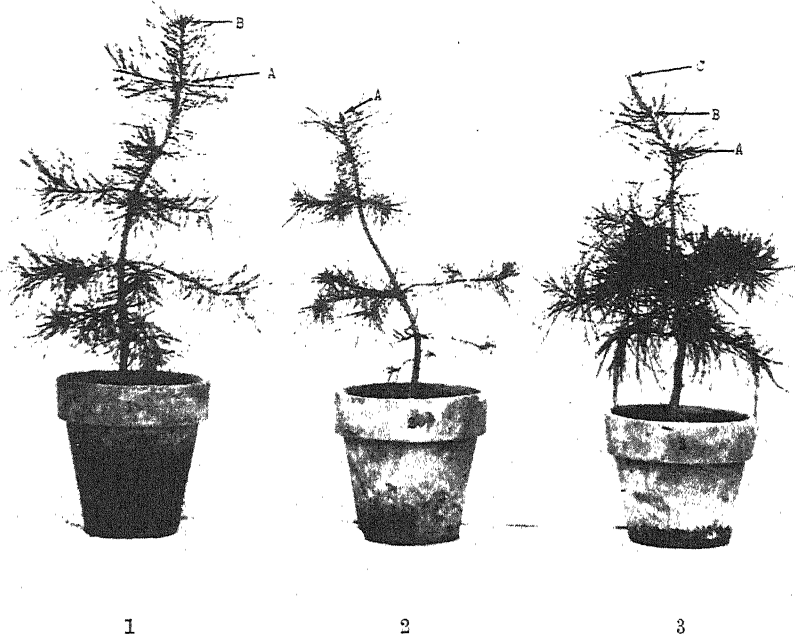


FIG. 1. *Pinus resinosa*. The three plants are of the same age and were treated alike until the fall of 1936 when plant 1 was placed in a cold frame and 2 and 3 left in a cool greenhouse. On March 5, plants 1 and 2 were brought into a warm greenhouse, and plant 3 was placed in another warm greenhouse and supplied with additional light, giving it a photoperiod of approximately 16 hours. Plant 1 grew the amount indicated between A and B; plant 2 did not grow at all; plant 3 went through two growth cycles (first it grew from A to B, after which, in the early summer, the distance B to C).

### Summary

It has been found that if *Pinus resinosa* seedlings are not exposed to freezing conditions during the winter, they make either no growth or only a very slight growth during the summer, unless exposed to a photoperiod of about 16 hours. If this is found to be true in nature, we would expect to find *Pinus resinosa* limited to regions where freezing occurs during the

winter. Some of the pine seedlings had two growth cycles when exposed to the long photoperiod.

The writer is interested in hearing from anyone who has first-hand information concerning the presence of *Pinus resinosa* in regions where there is no freezing temperature.

DEPARTMENT OF BOTANY  
UNIVERSITY OF MICHIGAN

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### EFFECT OF DROUGHT ON PROTOPLASMIC ELASTICITY<sup>1</sup>

HENRY T. NORTHERN

It is generally believed that the capacity of a plant to resist drought is determined to some extent by the nature of the protoplasm. The data in this paper indicate that the elasticity of protoplasm in cells of rye coleoptiles increases when moisture is deficient.

Seeds of spring rye were sowed in two flats. After sowing, both flats were thoroughly watered. The soil in one flat was then allowed to dry out, whereas the soil in the other flat was kept constantly moist. On the seventh and succeeding days after sowing, 6 plants were removed from each flat.

<sup>1</sup> Contributions from the Department of Botany and the Rocky Mountain Herbarium of the University of Wyoming, no. 165.



After the root systems and leaves which were emerging from the coleoptiles were cut off, the coleoptiles with enclosed leaves were centrifuged. Following centrifugation the responses of the chloroplasts in cells of the coleoptiles were noted. On the eighth and ninth days samples of soil were removed from each flat, weighed, allowed to dry at room temperature, and the moisture percentages on a dry weight basis were calculated. The data are summarized in table I, in which + indicates that the chloroplasts had been moved by the centrifugal acceleration whereas 0 indicates that the chloroplasts had not been moved.

TABLE I  
EFFECT OF DROUGHT ON PROTOPLASMIC ELASTICITY

DAYS AFTER SOWING	SOIL MOISTURE	LEAVES	RESPONSE OF CHLOROPLASTS TO CENTRIFUGATIONS OF			
			680 × GRAVITY FOR ONE MINUTE		170 × GRAVITY FOR FOUR MINUTES	
			IN CELLS NEXT TO VEIN	IN MORE DISTANT CELLS	IN CELLS NEXT TO VEIN	IN MORE DISTANT CELLS
7	Slightly dry Moist (control)	Turgid	+	+	+	+
7		“	+	+	+	+
8	5.9%	Slightly wilted	+	+	0	0
8	18.8 (control)	Turgid	+	+	+	0
9	2.8%	Slightly wilted	0	0	0	0
9	17.1 (control)	Turgid	+	+	+	+
10	Moist*	Turgid	+	0	+	0
10	Moist (control)	“	+	+	+	0
11	Moist*	Turgid	+	+	+	0
11	Moist (control)	“	+	+	+	0

\* After the experiment on the ninth day was completed the dry flat of plants was thoroughly watered.

NORTHERN (1, 2) has previously demonstrated that protoplasm in cells of *Zygnema* and *Spirogyra* is an anomalous fluid and hence is probably built up of intermeshed linear molecules or micelles. In *Spirogyra* the velocity with which the chloroplasts moved in response to centrifugal accelerations was approximately governed by the following equation:

$$V = k(c - c_0)$$

in which  $V$  is the velocity of chloroplastic movement,  $k$  a constant,  $c$  the centrifugal acceleration used, and  $c_0$  is the initial starting centrifugal acceleration at which, or below which, the chloroplasts will not move regardless of how long the acceleration is allowed to act.

The data in table I support NORTHERN's conclusion that protoplasm is an

anomalous elastic fluid. If protoplasm in cells of rye coleoptiles be a true fluid the chloroplasts should have been displaced as much with a centrifugation of  $170 \times$  gravity for 4 minutes as with a centrifugation of  $680 \times$  gravity for 1 minute. In several instances (table I) the chloroplasts were displaced in cells distant from the veins by an acceleration of  $680 \times$  gravity but were not displaced by an acceleration of  $170 \times$  gravity. In such instances the initial starting acceleration ( $c_0$ ) for such cells was equal to or greater than  $170 \times$  gravity. Hence when an acceleration of  $170 \times$  gravity was used:

$$V = k(170 - 170) = 0 \quad \text{or} \quad V = k(170 - > 170) = < 0.$$

In some instances, however, the chloroplasts were displaced by both accelerations. At such times the value of  $c_0$  was less than  $170 \times$  gravity. NORTHEN (2) has found that in *Spirogyra* the value of  $c_0$  varies from day to day.

The data in table I indicate that a deficiency of water caused an increase in the value of the initial starting acceleration ( $c_0$ ), which was interpreted as an increase in protoplasmic elasticity. For example, on the ninth day the value of  $c_0$  in cells with a deficiency of water was equal to or greater than  $680 \times$  gravity whereas for cells with sufficient water the value of  $c_0$  was less than  $170 \times$  gravity. Hence, for cells with a deficiency of water the velocity of chloroplastic movement when an acceleration of  $680 \times$  gravity was used would be:

$$V = k(680 - 680) = 0 \quad \text{or} \quad V = k(680 - > 680) = < 0.$$

However, for plants with sufficient water the velocity would be:

$$V = k(680 - < 170) = k(> 510).$$

The higher the value of  $c_0$  the greater the elasticity of the protoplasm and hence the more gel-like would be the protoplasm. Furthermore, it is highly probable that the more gel-like the protoplasm the more avidly it will retain water.

It will be noted (table I) that the changes in elasticity occurred in a relatively short time. For example, the elasticity of the protoplasm had returned to normal in cells adjacent to the veins 24 hours after the plants were watered and in all cells 48 hours after watering. The rapidity with which the changes in elasticity occurred suggest that in this experiment the changes in elasticity were due to orientations of existing molecules or micelles rather than to the formation of new substances or the increase in the amount of such substances as hemicelluloses.

UNIVERSITY OF WYOMING,  
LARAMIE, WYOMING

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## NOTES

**Annual Election.**—The fifteenth annual election of the American Society of Plant Physiologists, held during May and June, 1938, resulted in the choice of the following officers for 1938-1939: President, Dr. WALTER F. LOEHWING, State University of Iowa; vice president, Dr. GEORGE W. SCARTH, McGill University; elected member of the executive committee (term to expire in 1941), Dr. E. J. KRAUS, University of Chicago; elected member of the editorial committee (term to expire in 1941), Dr. EDWIN C. MILLER, Kansas State Agricultural College. Many opportunities for service will be presented to the membership of the Society, and the officers will appreciate the cooperation of all members in promoting the welfare and objectives of the organization. The immediate objective is preparation for the Richmond meeting in December, 1938. Prompt response to requests for service in connection with programs and committee assignments will lighten the tasks of those responsible for the program as a whole.

**New England Section Meeting.**—Although the New England Section held its meeting far from the center of distribution of its membership, and some members had to travel several hundred miles to attend, yet the meeting on the Maine Campus at Orono, Maine, was one of the best meetings the section has had. The attendance equaled or possibly exceeded that of previous meetings, and afforded evidence of the cooperation and friendship which annual sectional meetings can establish. It was a pleasure to all attendants to participate in the hospitality of Vacationland.

In addition to the program of papers, there was a symposium on the physiology of aquatic plants, with Dr. C. W. MUENSCHER, of Cornell, and Dr. C. COTTAM, of the United States Biological Survey, as guest speakers. Also, a special meeting dealing with the physiological problems of blueberry culture preceded the general sectional activities.

A report by Dr. J. C. LYON, Dartmouth, on the symposium at Indianapolis on methods of teaching plant physiology, stimulated sectional interest in a continuation of this work by a committee of which Dr. LYON is chairman.

The section interested itself in the problems of chemurgy, and organized a committee with Dr. C. G. DEUBER as chairman to consider the objectives of chemurgy and the relation of plant physiology to the attainment of those objectives.

The annual dinner was held on the evening of May 13, in Merrill Hall. In addition to the address of welcome by President ARTHUR A. HAUCK, there was a color motion picture on the Wild Life of Maine by JOSEPH S. STICKNEY, Warden Supervisor of the State Department of Inland Fisheries and Game.

Officers for the following year were elected. Dr. HUBERT BRADFORD VICKERY, of the Connecticut Agricultural Experiment Station, being chosen as chairman of the section. Dr. CHARLES J. LYON is vice-chairman, and Dr. LINUS H. JONES was re-elected secretary-treasurer.

The meeting in May, 1939, is scheduled to be held in New Haven. With several years of most successful operation, the New England Section is firmly established as part of the scientific progress of the region.

**Western Section.**—The meeting of the Western Section of the American Society of Plant Physiologists, held with the Pacific Division of the A.A.A.S. in Balboa Park, San Diego, was particularly successful this year. A four-day program offered three sessions for submitted papers, four joint symposia, and several festive occasions. The sessions were held in the Floral Association Building, in cool, well-ventilated, amply equipped rooms.

The session of Tuesday morning, June 21, was devoted to submitted papers on auxins, bud inhibition, polar transport, autonomic exudation cycle, and responses to ethylene. In the afternoon, a joint symposium with the Western Society of Soil Science considered salinity problems: Salt absorption and transport, by D. R. HOAGLAND and T. C. BROYER; toxicity and accumulation of chloride and sulphate salts, by F. M. EATON; and the program of the U. S. Regional Salinity Laboratory, by O. C. MAGISTAD. This symposium on salt tolerance emphasized the growing importance of salinity problems in irrigation agriculture, and the part plant physiology must play in the solution of these problems.

On Wednesday morning plant invasion on the Pacific coast was the topic of a joint session with the Botanical Society. Starting with a discussion of early introductions as revealed by study of plant remains in ancient adobe bricks, G. W. HENDRY and J. N. BOWMAN gave a vivid portrayal of the influence of the coming of the white man on plant succession in the west. H. L. MASON described the source and distribution of recent plant invaders; and C. J. KRAEBEL discussed the use of introduced species in planting road grades and burned areas in forests. M. W. TALBOT directed attention to the extent to which introduced species have invaded valley and foothill pastures in California. In some instances the invaders may form 70 per cent. of the present vegetation. Some of the invaders are beneficial, others weedy and detrimental. W. W. ROBBINS discussed the weed losses of agricultural areas.

A symposium on cell wall structure, Wednesday afternoon, included papers by W. H. DORE, O. L. SPONSLER, JAMES BONNER, W. S. STEWART, and A. S. CRAFTS. The papers presented the development of cell walls from the simplest molecular building units to their final rôle in the functioning plant. Particularly interesting and valuable were the results of the use of modern chemical, x-ray, and polarization methods of studying cell wall structure and the relation of structure to the attendant growth processes.

The Thursday morning session included papers on weed control, toxicity of insecticides in the soil, and plant-soil relations. The afternoon symposium on progress in plant science had noteworthy summaries on plant hormones by F. W. WENT; permeability, by L. R. BLINKS; dynamics of photosynthesis by W. ARNOLD; and nature of viruses, by T. E. RAWLINS.

The final session on Friday dealt with plant biochemistry and mineral nutrition, and revealed the progress being made with reference to the rôle of metals in the nutrition of plants.

The dinner for plant physiologists was held Thursday evening. Officers for the following year are: Chairman, F. W. EATON; vice-chairman, J. R. FURR; secretary, A. S. CRAFTS.

**Life Membership Committee.**—The fifteenth award of the CHARLES REID BARNES life membership will be made at the Richmond meeting. According to the constitution and recent ruling by the executive committee, this award is to be made to some prominent foreign plant physiologist. The committee as announced by president LOEWING is constituted as follows: Chairman, Dr. R. H. TRUE, University of Pennsylvania; E. C. AUCHTER, U. S. Department of Agriculture; D. R. HOAGLAND, University of California; F. M. ANDREWS, Indiana University; and SAM F. TRELEASE, Columbia University.

**Stephen Hales Award.**—The sixth award of the STEPHEN HALES prize is also to be made during the annual meeting at Richmond. Dr. HUBERT BRADFORD VICKERY, of the Connecticut Agricultural Experiment Station, is chairman of the award committee, with Dr. C. A. SHULL, the University of Chicago, and Dr. K. V. THIMANN, Harvard, as members of the committee. By tradition the announcement of these awards is made at the annual dinner, which is thus made one of the happiest features of the social activities of the Society. It is hoped that all plant physiologists attending the meetings will find it possible to attend the annual dinner and share in the pleasures of that occasion.

**Florence Brown Charlton.**—With deep regret we record the death on March 31, 1938, of Mrs. Florence Brown Charlton, a member of the Society, wife of Mr. CLARENCE D. CHARLTON, at La Salle, Illinois. Mrs. CHARLTON was born in Chicago on December 4, 1904, and was educated in the Chicago public schools. After graduation at the Englewood High School, Chicago, in 1921, she matriculated at the University of Chicago, where she specialized in botany and plant physiology for 5 years. She was repeatedly awarded honors and scholarships for excellence of her work in the junior and senior colleges, and was graduated with the S.B. degree with Phi Beta Kappa honors in June, 1925.

Continuing her work in the graduate school, and majoring in plant physiology, she won her M.S. degree in 1926, and was elected to associate membership in Sigma Xi. On September 1, 1926, she became a graduate assistant at the Pennsylvania State College, which institution she served faithfully for 10 years. She was advanced to the rank of assistant in botany on October 1, 1927, and to an instructorship on July 1, 1928. Because of local rulings that persons above the rank of instructor could not obtain advanced degrees at the Pennsylvania State College while a member of the college staff, she remained in the instructorship rank, and worked for her Ph.D. degree, which was granted her posthumously at the June, 1938, convocation. She taught general botany, and assisted Dr. W. H. Popp in plant physiology, was always an excellent teacher, a thorough and hard-working student, a very capable investigator. She was joint author with Dr. Popp of several valuable contributions and reviews on ultraviolet and visible radiations as factors in plant response. Her thesis was published in the July, 1938, number of the *American Journal of Botany*.

She was a member of the Botanical Society of America, and the Pennsylvania Academy of Science, as well as of the American Society of Plant Physiologists.

In June, 1936, she resigned her position at the Pennsylvania State College to engage in a career of home-making, a career in which she found great happiness. To retain her interest in plant physiology, she had been engaged temporarily as assistant editor of *PLANT PHYSIOLOGY*, and gave very valuable assistance with the October number of 1937. In recognition of her unique qualifications for this work, her command of English, and her sense of organization, arrangements had been concluded to have her begin a permanent connection with the editorial office on July 1, 1938. Action had been approved by the executive committee at the Indianapolis meeting, looking toward this end.

The immediate cause of her death was failure to rally after a Caesarean operation on March 27 for the birth of a daughter. Blood transfusions were given, but failed to save her. She had a fine clear mind, was exceptionally competent in all of her services, possessed a rare and beautiful personality, and a character above any possible reproach. Such personalities can never be replaced in kind, and her loss is felt most keenly by all who had the privilege of knowing her and working with her. Our sympathy is extended particularly to her bereaved husband and his family.

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**Annual Review of Biochemistry.**—The preparation and publication of the Annual Review of Biochemistry is an outstanding achievement which places all biochemists and physiologists under a debt of gratitude to the

editors, reviewers, and publishers of this annual volume. Volume VII is somewhat smaller than its immediate predecessor because some of the reviews contemplated did not come through for publication. The majority of the reviews cover the same fields as were covered last year. This is as it should be, for we need the summaries of active fields repeated year after year. A few new subjects of timely interest are treated in this volume.

The reviews which are repeated, with or without subdivisions of the previous treatment, and with different authorship, are as follows: Biological oxidations and reductions, by L. MICHAELIS and C. V. SMYTHE; chemistry of the crystalline enzymes, by J. H. NORTHROP and R. M. HERRIOTT; the chemistry of the carbohydrates and glycosides, by E. F. ARMSTRONG; the chemistry of the acyclic constituents of natural fats and oils, by G. S. JAMIESON; the chemistry of amino acids and proteins, by M. BERGMANN and C. NIEMANN; the chemistry and metabolism of the compounds of phosphorus, by K. LOHMANN; carbohydrate metabolism, by H. E. HIMWICH; fat metabolism, by F. VERZAR; metabolism of amino acids and proteins, by H. A. KREBS; the hormones, by O. WINTERSTEINER and P. E. SMITH; the vitamin B group, by R. A. PETERS and J. R. O'BRIEN; vitamin C (ascorbic acid, cevitamic acid), by R. A. PETERS and H. W. DAVENPORT; the fat-soluble vitamins, by J. C. DRUMMOND; nutrition, by H. H. MITCHELL; the biochemistry of muscle, by E. LUNDSGAARD; the chemistry of bacteria, by W. H. PETERSON and M. J. JOHNSON. In addition, there are seven titles, as follows: The metabolism of creatine and creatinine, by K. THOMAS; acid-base metabolism, by J. SENDROY, Jr.; liver and bile, by A. C. IVY and L. A. CRANDALL, Jr.; animal pigments, by R. LEMBERG; the terpenes, saponins, and closely related compounds, by W. A. JACOBS and R. C. ELDERFIELD; organic insecticides, by F. B. LAFORGE and L. N. MARKWOOD; and growth regulators in the higher plants, by P. BOYSEN JENSEN.

The reviews are excellent surveys of the fields covered, and are complete enough to keep the reader who depends upon them well informed as to the current trends of research, and the philosophy of biochemical interpretation. Year by year the *Annual Review of Biochemistry* has made itself a dependable source of authentic information, and it is recognized as an invaluable and indispensable part of the equipment of the biochemist and physiological investigator. With author and subject indices, the new volume contains 571 pages and retails at \$5.00 per copy. It can be ordered from Annual Reviews, Inc., Stanford University, California. The press work by the Stanford University Press is excellent.

It seems appropriate to call attention now to the fact that an *Annual Review of Physiology* is to be added to the publication activities of Annual Reviews, Inc., with cooperation of the American Physiological Society. This will permit separation of the reviews that are primarily physiological from

those that are primarily biochemical in nature. It is hoped that the new departure will be as successful, and as useful to investigators and teachers as the *Annual Review of Biochemistry* has become.

**General Plant Physiology.**—A new text book of plant physiology comes from the press of Williams and Norgate Ltd., London. The author is E. C. BARTON WRIGHT, well known to his American friends as the author of *Recent Advances in Plant Physiology*, published by Blakistons in 1930 and 1933. The new *General Plant Physiology* is divided into three parts: The general physiology of the cell; metabolism; and growth, reproduction and irritability. The six chapter headings of the first part are: The scope of physiology; colloids and protoplasm; osmotic pressure and the water relations of the plant; permeability; transpiration; and the ascent of sap. The second part contains seven chapters: Catalysis and enzymes; photosynthesis; the fats; nitrogen metabolism; ash; the transport of solutes; and respiration. The final part has four chapters: Germination; growth; reproduction; and irritability and plant movements. There is a brief appendix dealing with pH, a general bibliography, and author and subject indices. The total number of pages is 539, and the price not including the 15 per cent. advalorem duty, is 15 shillings. It is hoped that American plant physiologists will extend their acquaintance with the author by using it in connection with courses in plant physiology, and by making it available in libraries for the use of students. It contains a great deal of information, and reflects the points of view developed in the English laboratories, where many very valuable contributions have originated. The author deserves encouragement in his attempt to provide a text based upon the more fundamental general physiological processes. Orders may be sent to Williams and Norgate Ltd., Great Russell St., London. An American agent may be announced later.

**Soilless Growth of Plants.**—It was inevitable that the lay interest in tray agriculture, or solution culture of vegetables, flowers, and fruits, would be capitalized in book form. We record the publication of *Soilless Growth of Plants*, by the Reinhold Publishing Corporation, New York. The authors, CARLETON ELLIS and MILLER W. SWANEY, are not primarily plant physiologists, but private laboratory chemists (Ellis Laboratories, Montclair, New Jersey). The language is too "popular" in places, and misrepresents the points of view developed in plant physiological laboratories. This, perhaps, is to be expected, but the public gets a distorted idea from reading books that are not scrupulously accurate at the same time that they are popular reading. The reviewer believes that it is not necessary to depart from accuracy to make plant behavior interesting to the public.

This work is intended to educate the public in regard to the principles



and practices of solution culture plant production. And the public does need educating, as is revealed by the case of a man who approached the writer and proposed to sink a fortune of \$25,000 in tray agriculture by using an old barn which could neither be heated nor lighted as the site of his operations! The difficulty is that chemical laboratories are selling salt combinations to a gullible fraction of the population, on the theory that soilless culture of plants is practicable for the ordinary individual.

There are eight chapters, as follows: Chemistry of plant life; growing in mineral aggregates; growing in water; household plant culture; commercial aspects; special chemicals; common detriments; and nutrient formulas.

It is written in entertaining style, and will no doubt have many readers. Those who contemplate using solution culture methods of growing plants should consult, at the same time, the agricultural experiment station bulletins which cover this subject with unvarnished accuracy. The statement by HOAGLAND and ARNON, issued in February 1938 by the University of California; Bulletin 636 of the New Jersey Station; and circular 232, of the Indiana Station are recommended as collateral reading. The News Letter of the Committee on the Relation of Electricity to Agriculture, number 17, June 1938, published at 58 East Washington St., Chicago, contains a series of papers from California, Wisconsin, Indiana, and New Jersey, explaining *Nutrient Solution Culture Methods* in an interesting manner.

The price of the ELLIS and SWANEY work is \$2.75, and it can be ordered from the publishers, if not obtainable at local book stores.

**General Physiology.**—The third edition of Dr. PHILIP H. MITCHELL's *General Physiology* has been issued by the McGraw-Hill Book Co. It has been extensively revised for this edition, especially with reference to excitation, contraction, chemical transmission, membrane structure, permeability, physiological oxidations, muscle chemistry, vitamins, hormones, etc. Intended for college students who may have but one course in physiology, the emphasis is largely upon animal physiology, although it is called general physiology. In many instances, however, the discussion applies just as well to plant cells as to animal cells, and is truly general. The reviewer considers it an excellent text for the group for which it has been prepared. Each of the 25 chapters closes with a literature list of monographs, reviews, and journal publications which serves as a guide to extensive reading. The work is worthy of attention by all who desire to keep abreast of the progress made in the field of general physiology. The quoted price is \$6.00 per copy.

**Laboratory Manual of General Physiology.**—To accompany the text book of general physiology just noted, a laboratory manual has been prepared by Dr. P. H. MITCHELL and I. R. TAYLOR. The manual contains 254 exercises, arranged in 21 chapters, the order of which follows the order of

presentation of the text. The experiments are outlined in concise form, and offer a great variety of laboratory techniques. A good teacher should have no difficulty in providing an intensely interesting and valuable course from the suggested work. Many more experiments are outlined than can easily be performed during a single year, so selection will be necessary. The work can be adapted thus the more readily to individual laboratory equipment. The price of the manual (142 pp.) is \$1.50 per copy.



FRANCIS ERNEST LLOYD

OCTOBER 4, 1868



# PLANT PHYSIOLOGY

OCTOBER, 1938

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## DIURNAL FLUCTUATION IN ROOT PRESSURE<sup>1</sup>

KARL A. GROSSENBACHER

(WITH FOUR FIGURES)

Diurnal cycles in root activity under constant conditions seem to have been first described by HOFMEISTER in 1862 (7). Other investigators have confirmed his findings (1, 11), but in recent years it has been concluded that cycles in pressure and exudation were due to temperature fluctuations (HEYL, 5). On the other hand, studies in this laboratory (9) have substantiated HOFMEISTER's original results, and WHITE (10) has reported a cycle in exudation of excised tomato roots (though he did not control the conditions under which he was working). Complete proof of the existence of autonomic cycles has recently been obtained by the author with plants grown and tested under controlled conditions (4).

Several theories have been developed to explain the general phenomena of root pressure and exudation. For the purpose of this discussion it is sufficient to point out that osmotic mechanisms have generally been used. SABININ (8) holds the view that a simple osmotic mechanism is sufficient.

The general problem is confused by the controversy over the parts played by the phloem and xylem in translocation and root pressure. Since these measurements extended over periods of several days it seems probable that sieve tubes were plugged (CRAFTS, 3) and that the phloem played little part directly in maintaining the observed pressures. Here it is assumed that the hydrostatic-pressure system involved is the xylem-manometer system.

In these studies, plants (*Helianthus*) were grown in culture solution with forced aeration, in a greenhouse. The solution concentration was low,  $\frac{1}{4}$  H— (see explanation later) and typically not renewed, so that the plants were vigorous and healthy at the time of experimentation and were comparable to the "low salt plants" of HOAGLAND and BROYER (6), with root systems capa-

<sup>1</sup> The experimental work on which this paper is based was carried out at the University of California (at Berkeley) through the cooperation of the Department of Botany and the Division of Plant Nutrition, during the summer months of 1937.

ble of very active absorption. On several occasions such root systems maintained pressure for 14 days, indicating that they were quite healthy, had large food stores, and were "starved" only in the technical sense of having a low salt content.

A fairly satisfactory experimental technique has been developed: Plants were grown in two-quart jars fitted with sintered glass aerators. Small bore (1-mm.) u-tube manometers were built so that they might easily be fastened to the side of the jar with heavy rubber bands (fig. 1). The small

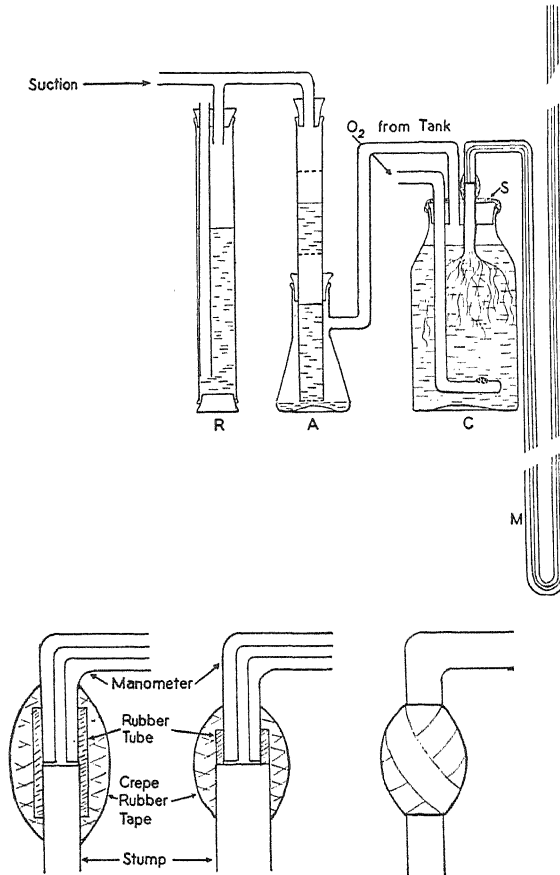


FIG. 1. Apparatus used to measure root pressure and respiration; R, regulator on suction line; A, absorption column; C, culture vessel (in which the plant was grown); S, paraffine seal; M, small bore mercury manometer.

bore and double action of the u-type manometer gave a convenient method of measuring pressure changes with only small change in volume of liquid in the xylem-manometer system (0.004 cc./cm. Hg). Manometers were attached to squarely cut stumps with the aid of rubber tubing and crepe rubber tape

("Sterilastic tape" carried by most drug stores). Such attachments could be made to withstand more than two atmospheres of pressure and resulted in a minimum of crushing or injury to stems. Except as designated, all solutions were balanced nutrient solutions, HOAGLAND's solution ( $\text{KH}_2\text{PO}_4$  0.001M;  $\text{MgSO}_4$  0.002M;  $\text{KNO}_3$  0.005M;  $\text{Ca}(\text{NO}_3)_2$  0.005M) served as a base. Concentrations are expressed as  $\frac{1}{4}$  H, 1 H, and 4 H, representing fractions or multiples of the original solution. During the growing period additions of iron and supplementary solution (boron, etc.) were made as needed. Plants grown in a greenhouse were transferred to a thermostatically controlled dark-room, and pressures were recorded at short intervals (usually 3 hours) under constant conditions of light, temperature, and aeration.

One of the most pronounced effects observed was a diurnal fluctuation in pressure which could not be correlated with any change in the environment of the plant during the test period. Careful study of thermograph records showed that there were slight shifts in temperature during extended periods but no diurnal fluctuation could possibly be read into the record for many of the days when root pressures showed such fluctuations.

When it became evident that root pressure did not reach an equilibrium under experimental conditions, considerable effort was made to secure as constant an environment as possible. Tanks of compressed oxygen were used to furnish a uniform supply of oxygen for the aeration of solutions. Artificial light at several intensities was used and remained as constant as commercial electrical service. These improvements did not measurably affect results and it is concluded that these plants were capable of maintaining a diurnal cycle in root pressure which was not directly related to current conditions but possibly controlled by a physiological cycle set up during the growth period in the greenhouse.

### Experimentation

Considerable variability was observed and not all plants showed such fluctuations (of special interest was a group of plants which had symptoms probably resulting from boron deficiency and which did not show a diurnal cycle). Data from three experiments are presented to illustrate some of the typical responses observed:

#### EXPERIMENT I

"Pure line" seed obtained from Cornell (S-24-150) was used. Plants were selected twice during their five-weeks growing period, and were quite uniform and vigorous. Setting up of the experiment began at 3 P.M. The solutions were changed to  $\frac{1}{2}$  saturated  $\text{CaSO}_4$  (*i.e.* 0.005M<sup>±</sup>) and  $\frac{1}{2}$  H, manometers attached, and aerators connected (tank  $\text{O}_2$ ), by 7 P.M. Absorption towers for  $\text{CO}_2$  were connected at 8 P.M. thus allowing a full hour for the solutions to come into equilibrium with the gas stream (the rate of gas flow was well above one liter per hour). Pressures were recorded every three

hours and respiration determined for six-hour (later twelve-hour) intervals. Data are presented graphically in figure 2, each curve representing the average

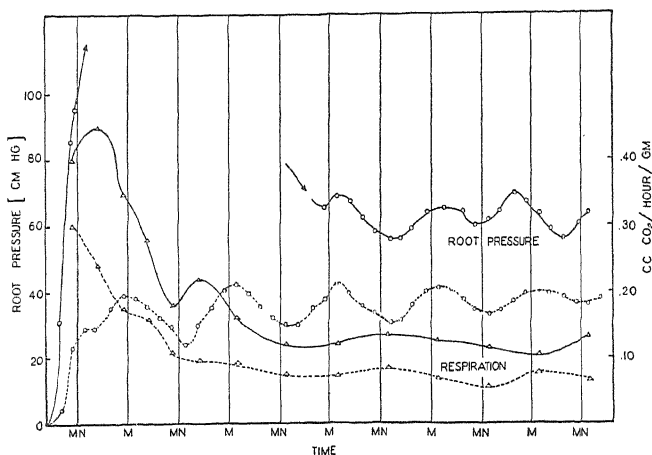


Fig. 2. Root pressure and respiration of 5-week-old sunflower plants. Solid lines represent averages obtained in balanced nutrient solution; broken lines represent averages obtained in dilute solution of  $\text{CaSO}_4$ .

for five plants. Values for individual plants were determined and plotted, but showed the same trends as the averages.

It is apparent from the curves that plants transferred to a fresh dilute, but balanced, nutrient solution respired appreciably more and developed much more root pressure than those transferred to dilute  $\text{CaSO}_4$ . In fact the manometers used did not have sufficient capacity (1 meter Hg) to measure the pressure produced by four of the five plants, and no significant measurement was made for a period of about forty-eight hours while mercury was being forced back into the manometers and allowed to come to equilibrium again. Subsequent readings may have been affected by the loss from the root systems of a considerable volume of exudate immediately following the "blowing out" of the manometers. Even with this treatment a fairly distinct diurnal fluctuation is evident in the root pressure values. Plants in dilute  $\text{CaSO}_4$  had lower root pressures than those in complete nutrient solution and they maintained a diurnal fluctuation well into the sixth day. In neither case did the plants show a consistent relation between root pressure and respiration. Respiration values for both groups dropped rapidly during the first twenty-four hours and then leveled off with the complete-solution group maintaining a 20 to 50 per cent. higher rate.

## EXPERIMENT II

Groups of three similar plants five weeks old were treated in the following manner, five days before the experiment was set up: The first group



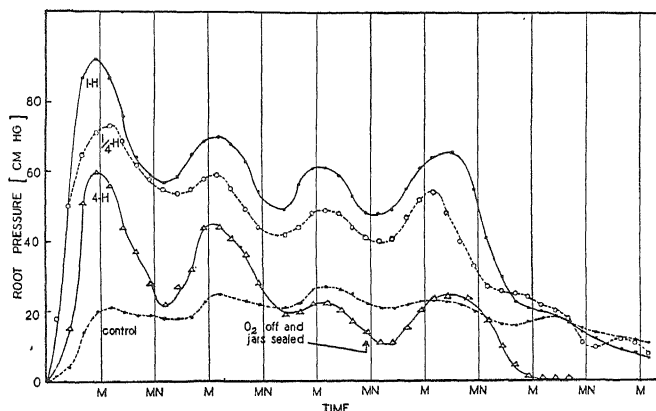


FIG. 3. The effect of concentration of balanced nutrient solution and oxygen supply on root pressure.

was left undisturbed (*i.e.*, the solution contained the unused portion of  $\frac{1}{4}$  H solution in which the plants were grown), while other groups received additional nutrient salts equivalent to  $\frac{1}{4}$  H, 1 H, and 4 H. Those treated with full strength solution showed the highest pressure. The untreated ones gave the lowest and most constant readings while those treated with 4 H developed a fairly high pressure but failed to maintain it. All four groups showed diurnal fluctuations.

After three days the oxygen was turned off (all groups of plants) and the jars sealed with paraffine. The curves (fig. 3) show that root pressure was definitely affected in about one day. By taking values from the previous respiration curve it was calculated that the root systems were using about 25 cc. of oxygen per day. The solution contained about 12 cc. (assuming saturation), and a small space between the solution and cork was filled with oxygen which, however, could move into the solution only very slowly, as there was no stirring, and convection currents would be greatly reduced in a thermostated room. Thus it is indicated that under these conditions *Helianthus* roots may maintain root pressure until the rate of oxygen supply is greatly reduced, but that the pressure falls when oxygen becomes too low or the carbon dioxide partial pressure too high.

### EXPERIMENT III

The root pressure of four groups of three plants each were measured for two days and then additions were made equivalent to  $\frac{1}{4}$  H, 1 H, and 4 H, and readings taken at short intervals (fig. 4). It should be noted that the time scale is greatly increased, and the changes are rapid. In the three cases in which additional salts were used, a sharp decline in pressure was observed; its magnitude, however, was not quantitatively related to the osmotic value of added salts. In less than two hours all pressures were rising rapidly.

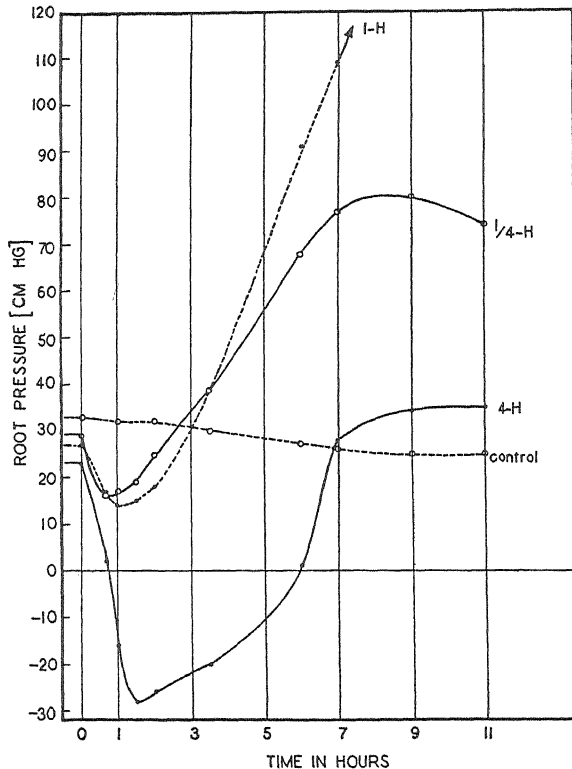


FIG 4. The effect of addition of nutrient salts on root pressure (addition made two days after manometers were attached).

In seven hours all reductions had been completely overcome. Again full strength solution gave maximum response, forcing the mercury out of the manometers. The 1 H solution used has an approximate osmotic value of 0.7 atmosphere. If we assume for convenience that its actual value is 50 cm. of Hg we see that the reduction produced by the  $\frac{1}{4}$  H solution was in the same order as that expected as a result of simple osmosis but that the reductions produced by either 1 H or 4 H solution was only  $\frac{1}{4}$  of that expected.

### Discussion

An explanation of root pressure in terms of a *simple* osmotic mechanism seems impossible in the presence of a diurnal cycle because: first, it would be necessary to assume that solutes (organic or inorganic) were periodically released into and removed from the sap; second, since the volume of liquid in the xylem-manometer system changes in the same way that the pressure changes, it follows that water must be entering and leaving at the same time solutes do; third, to be effective osmotically, changes in solutes must occur in the same regions in which water enters and leaves.

When electrolytes are added to the external solution (exp. III) there is an apparent osmotic effect (the sudden decrease in pressure). A stimulating effect of the added salts soon overcomes this in the case of active plants and builds the pressure up again. The first response may in reality be simply osmotic. The subsequent rapid rise in pressure must be the result of water entering the xylem system, and if this water enters in response to osmotic force it must be entering while the salts are becoming continuously more concentrated.

From these considerations it seems clear that if we assume a *simple* osmotic mechanism to be responsible for these changes in root pressure we are forced to admit:

- (1) that water and solutes must enter and leave the xylem system together, *i.e.*, as a solution.
- (2) That such movement cannot be due to osmotic force and hence our original premise is incorrect.

Root pressure, then, is not caused simply by the difference in osmotic values of the xylem sap and the external solution, but must be caused by some other forces in the root system which control and activate this movement. Evidently the mechanism of the diurnal cycle is in some way related to the mechanism responsible for root pressure. Yet, it has long been established that under many conditions there is a relation between root activity and respiration. Several such correlations are apparent in these experiments but the diurnal cycle of root pressure was not shown to be paralleled by a cycle in respiration. If such a cycle exists, its magnitude is probably less than that of root pressure.

Changes in permeability might explain fluctuations in exudation alone (8) but will not explain pressure fluctuations in a simple osmotic system since they would affect only the rate of change of pressure and not its equilibrium values.

If, however, a complex osmotic system be postulated the above reasoning breaks down. It may be argued that with increasing pressures solutes are released into the xylem sap in localized areas (cells or parts of cells) and that water enters in response to osmotic force in the immediate vicinity, while with decreasing pressure solutes could either be reabsorbed or merely diffuse out. In this case the cycles could be explained as a shifting balance between the two opposing processes. This is not, however, a simple osmotic mechanism and it would be impossible to speak of the osmotic value of the internal solution as it would in reality consist of a whole series of concentration gradients being constantly upset by diffusion and mass flow of sap resulting from movement of water and changes in pressure. It would be exceedingly difficult to obtain direct evidence to prove or disprove such a theory.

BENNET-CLARK, GREENWOOD, and BARKER (2) and others have concluded

that in potential growing tissues there is "positive water secretion" from the protoplasm into the vacuole in addition to the "osmotic pressure of the vacuole." Whatever the mechanism of such secretion is, it is becoming increasingly clear that some such process must be involved in many phases of the movement of water in living tissues. In root pressure it seems probable that such a force may operate in either direction and will, in addition to the osmotic forces involved, determine the pressure.

It would be pretentious to list those who have in some way contributed to this brief paper, but I wish to thank them for their help, especially for criticism of the discussion.

THE UNIVERSITY OF CALIFORNIA  
BERKELEY, CALIFORNIA

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# FOLIAR DIAGNOSIS: ITS RELATION TO THE OPTIMUM NUTRITION OF THE POTATO<sup>1</sup>

WALTER THOMAS  
(WITH SEVEN FIGURES)

## Introduction

The principles and application of the method of foliar diagnosis have been described in detail in a recent paper (8) using as illustrative material potato plants growing on seven plots of tier 1, section B, of the long continued Vegetable Fertility Plots of the Department of Horticulture of the Pennsylvania Agricultural Experiment Station. The plots examined in the previous paper, familiarity with which is assumed in the present paper, were:—N (plot no. 2), P (plot no. 3), K (plot no. 4), NP (plot no. 6), NK (plot no. 7), PK (plot no. 8), and NPK (plot no. 10).

The yields of tubers from the highest yielding plots previously reported, *viz.*, NK (plot no. 7) and NPK (plot no. 10), were considerably below the yields from the manure plot (no. 15) and were also below the yields from certain plots from the adjacent tier 2, which received complete mineral fertilizers also, but differed in the quantities and ratios applied. The plot (no. 15) treated with rotted horse manure at the rate of 30 tons per acre (600 lb. per 1/100-acre plot) has consistently outyielded the other plots. The plants growing on this manure plot which have shown good development throughout the season, accordingly, may be regarded as well nourished and at or nearing the optimum for the soil and climatic conditions prevailing in this region (7).

In this paper the relation of the foliar diagnosis of the best commercial fertilizer treatments to that of this manure plot (plot no. 15, tier 1) will be examined.

## Materials and methods

### PLOTS WITH TREATMENTS AND YIELDS

The experimental plan as described by MACK (1) is as follows:—"The area is laid out in four sections, 245.8 feet wide and 308.4 feet long ..... Each of the sections is divided into six tiers, ..... The tiers are further divided into seventeen plots, the dimensions of which are 12 by 36.3 feet, and which are separated from each other by guard strips 6 feet wide. The area of the plots, exclusive of guard strips, is exactly 1/100 of an acre."

Table I lists the plots examined and discussed in this paper together with

<sup>1</sup> Authorized for publication as Paper no. 795 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

their fertilizer treatment and yields of tubers for the year 1935. The treatment and yields of the plots reported in the first paper of this series (8) also are given for comparison.

TABLE I  
PLOT NUMBERS, TREATMENTS, AND YIELDS OF TUBERS

TIER	PLOT	TREATMENT	AMOUNT APPLIED TO PLOT	N, P <sub>2</sub> O <sub>5</sub> , AND K <sub>2</sub> O EQUIVA- LENT	SYMBOL AND RATIO	YIELDS*
			lb.	lb.		lb.
1	15	Rotted horse manure .....	600†	4.62 3.08 3.78	15: 10: 12.3‡	196
2	8	{ Nitrate of soda .....	4.0	0.6	N(1.5P)K 6: 15: 8	176
		{ Superphosphate .....	9.375	1.5		
		{ Muriate of potash .....	1.666	0.8		
2	12	{ Nitrate of soda .....	4.0	0.6	NP(1.5K) 6: 10: 12	174
		{ Superphosphate .....	6.25	1.0		
		{ Muriate of potash .....	2.499	1.2		
2	16	{ Nitrate of soda .....	8.0	1.2	2(NPK) 12: 20: 16	170
		{ Superphosphate .....	13.5	2.0		
		{ Muriate of potash .....	3.332	1.6		
1	7	{ Nitrate of soda .....	4.0	0.6	NK 6: 0: 8	163
		{ Muriate of potash .....	1.666	0.8		
1	10	{ Nitrate of soda .....	4.0	0.6	NPK 6: 10: 8	162
		{ Superphosphate .....	6.25	1.0		
		{ Muriate of potash .....	1.666	0.8		
2	4	{ Nitrate of soda .....	6.0	0.9	(1.5N)PK 9: 10: 8	157
		{ Superphosphate .....	6.25	1.0		
		{ Muriate of potash .....	1.666	0.8		
1	4	Muriate of potash .....	1.666	0.8	K	155
2	14	{ Nitrate of soda .....	2.0	0.3	0.5(NPK) 6: 10: 8	150
		{ Superphosphate .....	3.125	0.5		
		{ Muriate of potash .....	0.833	0.4		
1	8	{ Superphosphate .....	6.25	1.0	PK 0: 10: 8	148
		{ Muriate of potash .....	1.666	0.8		
1	7	{ Nitrate of soda .....	4.0	0.6	NP 6: 10: 0	124
		{ Superphosphate .....	6.25	1.0		
1	3	Superphosphate .....	6.25	1.0	P	114
1	2	Nitrate of soda .....	4.0	0.6	N	109

\* The yields are per plot of 1/100 acre each.

† Containing 65.7 per cent. moisture.

‡ Ratio is on a basis of percentage in 1000 lb. per acre of complete fertilizer.

#### RATIOS OF N, P<sub>2</sub>O<sub>5</sub>, AND K<sub>2</sub>O IN THE FERTILIZERS APPLIED

The fertilizer applied to plot no. 10, tier 1, has been designated the "standard" fertilizer (1). The "standard" fertilizer applied in the pres-

ent experiments at a rate such as to apply 60, 100, and 80 lb. of N,  $P_2O_5$ , and  $K_2O$ , respectively, per acre is commonly designated as a 6:10:8 fertilizer reflecting the ratios in which the "entities" are present. The amounts and ratios of N: $P_2O_5$ : $K_2O$  used on the other plots are given in table I.

The ratios given in table I for the horse manure are not comparable with the ratios given for the commercial fertilizers. There is no known chemical *in vitro* way of determining the availability of N,  $P_2O_5$ , and  $K_2O$  in manure. The amounts and ratios given in table I for manure assume complete availability of all the N,  $P_2O_5$ , and  $K_2O$  present. This is not the case. Very little of the phosphoric acid and nitrogen is soluble in water, although nearly the whole of the potash may be. On the other hand, the amounts of phosphorus and potash fixed by the soil from manure is negligible in comparison with the amounts fixed from the salts present in commercial fertilizers. The availability of the commercial fertilizer added to a soil is contingent upon many properties of the soil (2, 4, 5, 6).

Since all the plots examined in this paper have received as dressings all three elements—nitrogen, phosphorus, and potassium—the mode of nutrition of well-nourished plants is being explored. The physiological differences examined, therefore, are those of plants the yields of which are in the higher ranges, whereas in the previous paper (8) the physiological differences examined were those of plants the yields of which ranged from poor to good.

#### SAMPLING OF THE LEAVES

This was carried out in the same manner as previously described. For a complete discussion of method and principles of sampling the earlier paper (8) should be consulted, but for convenience a brief outline is presented herewith. Leaves of the same rank (physiological or metabolic age) were taken at each sampling date from all of the plants along a row lengthwise of each plot. Successive rows were then sampled in like manner on each successive sampling date. The leaves chosen from each plant were those immediately above the caducous leaves at the base of a stalk.

In the present experiment the fourth and fifth leaves were taken from two stems issuing directly from the mother tuber of the fourteen plants constituting a row. The samplings were carried out on four dates during July and August, 1935, consecutive rows in each plot being sampled on each successive sampling date.

It has already been pointed out (8) that the number of samplings is arbitrary, but at least four should be taken. The number of leaves sampled from each plant also is immaterial but the number should not differ too much from one plant to another. The development of the plants on each plot should be homogenous so that the leaves selected are representative of the plants on the plot.

### Presentation of results

The results of the analysis of the leaves constituting the foliar diagnosis are shown in table II expressed as (a) percentages of N,  $P_2O_5$ , and  $K_2O$  in the dried foliage, (b) milligram equivalents, and (c) the *NPK-unit* (i.e., the  $N-P_2O_5-K_2O$  equilibrium).

It is again emphasized that the graph for any treatment is an analytical characteristic of the chemical condition (state) of the selected leaf of plants growing on that plot at the time of sampling. The chemical condition is referred to as the percentage of dry matter in the leaf. Neither the number of leaves sampled from each plant nor the weight of the dry matter are taken into consideration in the method of foliar diagnosis.

### Discussion and interpretation of results

#### NPK-UNIT

The *NPK-unit* concept and the method of derivation has been given in an earlier paper (8).

The *NPK-unit* represents the equilibrium between nitrogen, phosphoric acid, and potash in the leaf considered at the moment of sampling. The manner of derivation of the *NPK-unit* indicates that it is independent of the values of *s* (column 5, table II), and which represents the total quantity (or *intensity*) of nutrition at the moment of sampling.

Consequently, the *NPK-unit* is a magnitude which expresses the *quality* of nutrition as distinguished from the *quantity* factor (*s*). However, although these two magnitudes are abstractly distinct, they are inseparably connected in the physiological processes of the leaf and must, therefore, be considered simultaneously.

In table II the composition of the *NPK-unit* is represented by the values of X, Y, and Z, shown in the last three columns. For each simultaneous value of X, Y, and Z there is one point and only one point on an equilateral triangle of sides = 100.

#### INDICATIONS GIVEN BY THE GRAPHS

GRAPHIC REPRESENTATION WITH PERCENTAGE OF ELEMENTS AS ORDINATE AND DATES OF SAMPLING AS ABSCISSA.—Figure 1 shows the graphic representation of the foliar diagnosis of the plants growing on these plots with the percentage of N,  $P_2O_5$ , and  $K_2O$  as ordinate and the dates of sampling as abscissa.

The facts brought out from an examination of the plants growing on the plots reported in the previous paper (8) are true also for the plants growing on the plots examined in the present paper. The graphs (foliar diagnosis) of the differently fertilized plots which have given diverse yields show a regularity which is related to the nature of the different fertilizers in that



TABLE II

COMPOSITION OF LEAVES OF RANK 4 AND 5 AT PERIODIC INTERVALS IN TERMS OF PERCENTAGE OF N, P<sub>2</sub>O<sub>5</sub> AND K<sub>2</sub>O IN DRIED FOLIAGE, MILLI-GRAM EQUIVALENTS, THE NPK-UNIT, AND THE SUM (N + P<sub>2</sub>O<sub>5</sub> + K<sub>2</sub>O)

DATE OF SAMPLING	DRIED FOLIAGE				MILLIGRAM EQUIVALENTS				COMPOSITION OF THE NPK-UNIT			
	N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	M <sub>x</sub> + M <sub>y</sub> + M <sub>z</sub>	N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	(S)	X	Y	Z	
	(M <sub>x</sub> )	(M <sub>y</sub> )	(M <sub>z</sub> )	(s)	(E <sub>x</sub> )	(E <sub>y</sub> )	(E <sub>z</sub> )	E <sub>x</sub> + E <sub>y</sub> + E <sub>z</sub>	(100 $\frac{E_x}{S}$ )	(100 $\frac{E_y}{S}$ )	(100 $\frac{E_z}{S}$ )	
	%	%	%	%	mg. eq.	mg. eq.	mg. eq.	mg. eq.	%	%	%	
	Manure plot (tier 1, no. 15)											
July 7	4.65	0.688	7.550	12.888	332.010	29.102	160.815	521.927	63.612	5.575	30.812	
July 29	3.80	0.608	5.138	9.536	271.320	25.718	109.226	406.264	66.785	6.331	26.885	
Aug. 9	3.79	0.564	5.155	9.509	270.606	23.357	109.801	404.264	66.939	5.902	27.161	
Aug. 24	3.28	0.448	6.658	9.386	234.192	18.950	120.515	373.657	62.075	5.071	32.254	
	N(1.5 P)K plot (tier 2, no. 8)											
July 7	4.84	0.710	6.969	12.519	345.576	30.033	148.440	524.049	65.944	5.730	28.325	
July 29	3.72	0.564	4.914	9.198	256.608	23.857	104.668	394.133	67.391	6.054	26.557	
Aug. 9	3.46	0.482	4.934	8.876	247.044	20.389	105.094	372.527	66.314	5.473	28.209	
Aug. 24	2.86	0.350	4.670	7.880	204.204	14.805	99.471	318.480	64.117	4.650	31.233	
	NP(1.5 K) plot (tier 2, no. 12)											
July 7	4.86	0.594	7.798	13.252	347.004	25.126	166.097	538.227	64.470	4.669	30.860	
July 29	4.19	0.522	5.737	10.479	299.166	22.081	122.837	444.084	67.368	4.972	27.661	
Aug. 9	3.71	0.513	5.782	10.005	264.894	21.699	123.157	409.750	64.647	5.286	30.057	
Aug. 24	2.82	0.394	5.996	9.210	201.348	16.666	127.715	345.729	58.239	4.822	36.939	
	2(NPK) plot (tier 2, no. 16)											
July 7	5.23	0.682	8.771	14.683	373.422	28.849	186.822	589.093	63.389	4.897	31.713	
July 29	4.04	0.562	5.930	10.532	288.456	23.773	126.309	438.538	65.777	5.420	28.802	
Aug. 9	3.87	0.500	6.134	10.494	276.318	21.150	130.441	427.909	64.574	4.943	30.483	
Aug. 24	3.30	0.354	6.453	10.107	235.620	14.974	137.449	388.043	60.720	3.858	35.422	

TABLE II—(Continued)

DATE OF SAMPLING	DRIED FOLIAGE				MILLIGRAM EQUIVALENTS						COMPOSITION OF THE NPK-UNIT			
	N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	M <sub>x</sub> + M <sub>y</sub> + M <sub>z</sub>	N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O	E <sub>x</sub> + E <sub>y</sub> + E <sub>z</sub>	X	Y	Z			
	(M <sub>x</sub> )	(M <sub>y</sub> )	(M <sub>z</sub> )	(s)	(E <sub>x</sub> )	(E <sub>y</sub> )	(E <sub>z</sub> )	(S)	$\left(100 \frac{E_x}{S}\right)$	$\left(100 \frac{E_y}{S}\right)$	$\left(100 \frac{E_z}{S}\right)$			
	%	%	%	%	mg. eq.	mg. eq.	mg. eq.	mg. eq.	%	%	%			
	NPK plot (tier 1, no. 10)													
July 7	4.98	0.584	6.589	12.153	355.57	24.70	140.34	520.62	68.30	4.74	26.96			
July 29	3.88	0.509	4.337	8.726	277.03	21.53	92.38	390.94	70.86	5.51	23.63			
Aug. 9	3.62	0.482	4.697	8.799	258.47	20.39	100.05	376.90	68.22	5.38	26.40			
Aug. 24	3.14	0.392	4.348	7.880	224.19	16.58	92.61	333.39	67.25	4.97	27.78			
	(1.5 N)PK plot (tier 2, no. 4)													
July 7	5.23	0.614	6.780	12.624	373.422	25.972	144.414	543.808	68.667	4.775	26.555			
July 29	4.00	0.488	5.040	9.528	285.600	20.642	107.352	413.594	69.054	4.990	25.956			
Aug. 9	3.98	0.464	4.534	8.978	284.172	19.627	96.574	400.373	70.977	4.903	24.120			
Aug. 24	3.16	0.356	4.302	7.818	225.624	15.059	91.633	332.316	67.892	4.532	27.572			
	0.5 (NPK) plot (tier 2, no. 14)													
July 7	4.84	0.520	6.108	11.468	345.576	21.996	130.100	497.672	69.439	4.418	26.142			
July 29	3.77	0.528	4.379	8.677	269.178	22.334	93.273	384.785	69.957	5.803	24.239			
Aug. 9	3.34	0.466	4.031	7.837	238.476	19.712	85.860	344.048	69.315	5.729	24.956			
Aug. 24	2.84	0.418	3.920	7.178	202.776	17.681	83.496	303.953	66.715	5.817	27.468			

(1) the percentages of nitrogen, phosphoric acid, and potash found in the dried foliage reflect the differences in the quantity of the particular element applied in the sense that response to a particular element (as deduced from the development and yield) is shown by a corresponding increase in the percentage of that element in the dried foliage; (2) the graphs for nitrogen, phosphoric acid, and potash, respectively, show by their relative forms and relative positions with respect to the corresponding graphs in the other plots the difference in the nature ("chemism") or course of nutrition with respect to these elements which is correlated with the different fertilizers.

Thus differences in the slopes of two graphs from one sampling date

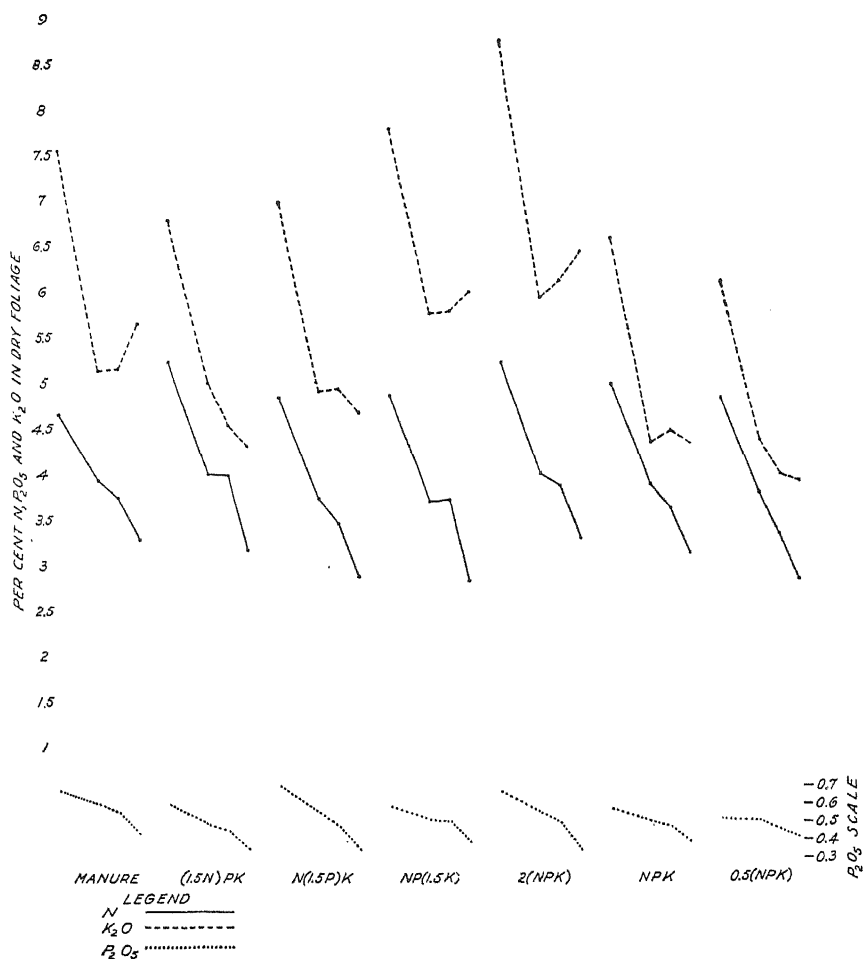


FIG. 1. Values of the dominant elements (expressed in percentage of the dried foliage) at different sampling dates in leaves from plants receiving the different treatments indicated.

to the next indicate differences in the relations of demand for a particular element by the plant to supply of that element to the plant. Hence, if the graph of an element does not sensibly decrease with time, this fact would indicate that the particular element is in relative excess or "bottled up" in the leaf.

*Characteristics of the graphs.*—(a) *Nitrogen graphs.*—The graphs of the 2(NPK) plot (no. 2-16) and the (1.5 N)PK plot (no. 2-4), both of which received higher nitrogen applications than the other plots, are higher than the other graphs during the first two periods correlated with which is a higher development of the aerial parts. This higher content of nitrogen, however, has not resulted in giving the highest yields of tubers.

The nitrogen graphs of the 0.5(NPK) plot (no. 2-14), the N(1.5 P)K plot (no. 2-8), and the NP(1.5 K) plot (no. 2-12) are similar in position but not always in form. The position of the nitrogen graph of the NPK plot (no. 1-10) is (as we should predict from the facts of the reciprocal effects of the elements on the absorption of nitrogen) intermediate to the graphs of 2(NPK) and (1.5 N)PK on the one hand, and of N(1.5 P)K and NP(1.5 K) on the other.

The graph for the highest yielding plot—the manure plot (no. 1-15)—is unique. The graph for nitrogen is lower than that for any of the above plots at the first sampling date (July 7), but higher than any except that of the 2(NPK) plot at the last sampling date (August 24).

As already stated, the relative steepness (slope) of the graphs is an index of the relative rates of demand and supply of the particular element, a very steep descent indicating a greater demand on the selected leaf relative to supply. The relatively small slope, therefore, of the nitrogen graph of the manure plot (no. 1-15) suggests that the supply, although not at a very high level at the beginning of the sampling (July 7) relative to some of the other plots, continuously keeps pace with the demand by the plant throughout the whole period.

Although 2(NPK) (no. 2-16) contains twice as much nitrogen as that of the "standard" fertilizer NPK, the increase in yield has not been notable and in (1.5 N)PK (no. 2-4) an actual decrease in yield is recorded compared with the "standard" fertilizer. And at the beginning of vegetation the nitrogen contents of 2(NPK) (no. 2-16) and (1.5 N)PK (no. 2-4) are identical.

It would be correct accordingly to conclude that since a content of nitrogen in the leaf in 1935 of 4.86 per cent. was sufficient to give the highest yields obtained, that the content of 5.35 per cent. in 2(NPK) (no. 2-16) is in excess, but that the buffer capacity of the plant has prevented any injury to the yield in 2(NPK) (no. 2-16) but may have been injurious in (1.5 N)PK (no. 2-4) on account of reducing the  $P_2O_5$  content of the leaf.

There is accordingly a relationship between the quantity of nitrogen in the leaf and nitrogen in the fertilizer, but there is no relationship between the quantity of nitrogen in the leaf and the yield nor between nitrogen in the fertilizer and the yield.

(b) *Phosphoric acid graphs.*—The graphs for the N(1.5 P)K plot (no. 2-8), the 2(NPK) plot (no. 2-16) and the manure plot (no. 1-15) are higher than those of any of the other plots during the first two periods, after which greater demand relative to supply causes the graphs of these three plots to fall below those of three other plots. The graphs of the aforementioned plots are similar in form except that the slope of the graphs for the manure plot (no. 1-15) shows the same characteristic as that of the nitrogen graph for this plot, *viz.*, a high level of supply adequate for the demand throughout the whole period.

The graphs for the NPK plot (no. 1-10) and that for the NP(1.5 K) plot (no. 2-12) have nearly the same form and position.

On the other hand, the graph for the (1.5 N)PK plot (no. 2-4) descends below that of the NPK graph (no. 1-10), suggesting that the increment of nitrogen has occasioned a decrease of phosphoric acid in the leaf resulting in a reduction of the yield.

It is significant in this connection to observe that an increment of phosphoric acid in the fertilizer and reflected in the leaf [*cf.* N(1.5 P)K (no. 2-8) with NPK (no. 1-10)] and also an increment in the potash in the fertilizer and also reflected in the leaf [*cf.* NP(1.5 K) (no. 2-12) with NPK (no. 1-10)] have in both cases resulted in increased yields by reducing the nitrogen content of the leaf.

In 1935 a content of phosphoric acid in the dried leaf of 0.68 per cent. in the early stages of vegetation has been sufficient for maximum growth. In N(1.5 P)K (no. 2-8) the content of  $P_2O_5$  has somewhat exceeded this without detriment, however, to the yield.

Accordingly, there is a relationship between phosphoric acid in the leaf and its presence in the fertilizer. There is, however, no relationship between phosphoric acid in the leaf and the yield and none between the quantity of phosphoric acid in the fertilizer and the yield.

(c) *Potash graphs.*—The graphs for the 2(NPK) plot (no. 2-16), the NP(1.5 K) plot (no. 2-12), the manure plot (no. 1-15), and the N(1.5 P)K plot (no. 2-8) are higher than any of the other plots throughout the whole period. It is significant, too, that these four plots are the highest yielding plots in tiers 1 and 2. The graphs for the three first mentioned plots are similar in form but not in position. The relatively very steep slopes of the graphs of the 2(NPK) plot (no. 2-16) and also of the manure plot (no. 1-15) during the first period indicate a greater demand on the potash relative to supply to the plants growing on these plots. Commencing with the

second period, accumulation of potash ("luxuskonsumption") (3) occurs in the leaves of plants growing on these plots.

In the commercial fertilizer plots a content of potash of 7.79 per cent. in the dried foliage at the earlier stages of vegetation has been sufficient for maximum yields. The potash content of the leaves of 2(NPK) has exceeded this limit by about 1 per cent., but has not resulted in a serious reduction in the yield.

The buffer capacity of the plant, therefore, is very great with respect to potash in plants receiving all three elements, nitrogen, phosphorus, and potassium. This is probably one reason for the success of complete fertilizers, although they may not be properly equilibrated.

There is, accordingly, a relationship between potash in the leaf and potash in the fertilizer, between the quantity of potash in the leaf and the yield, and between potash in the fertilizer and the yield.

In the earlier paper (8) it was pointed out that the basic assumption in field plot experiments is that the factors other than those introduced are nearly equal. On this basis the development and yield is related to the composition of the leaf with respect to nitrogen, phosphoric acid, and potash. The equilibrium between these elements will now be examined.

GRAPHIC REPRESENTATION OF THE COURSE OF NUTRITION AS SHOWN BY THE RESPECTIVE COMPOSITE *NPK-UNITS*.—The relationship of the  $\text{N-P}_2\text{O}_5\text{-K}_2\text{O}$  equilibrium to yields of tubers is readily shown by means of graphs in which the *NPK-unit* is plotted at each successive sampling date in trilinear coordinates.

The derivation of the composite *NPK-unit*, which indicates the equilib-

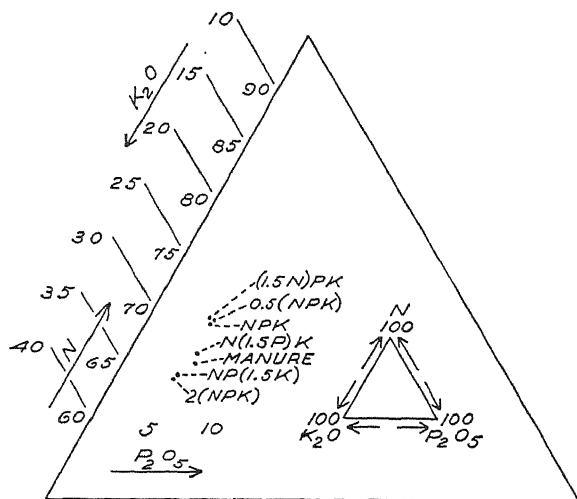


FIG. 2. Mean  $\text{N-P}_2\text{O}_5\text{-K}_2$  equilibrium (mean *NPK-units*) of leaves from plants receiving the different treatments indicated.

rium between  $N\text{-}P_2O_5\text{-}K_2O$  at the moment of sampling, has been given in an earlier paper (8). The composition of the *NPK-units* at each sampling date for each of the plots is given in the last three columns of table II.

*Mean NPK-unit and mean intensity of nutrition.*—The relationships to each other of the *NPK-units* of leaves from plants growing on the respective plots can be shown at a glance by means of a point (fig. 2). Any one point is the coordinate of the mean *NPK-units* at the successive sampling dates of the fourth leaf from plants growing on a particular plot. These points are, therefore, the mean values of X, Y, Z given in table II and correspond to the center of gravity (C.G.) of the detailed diagrams given in figures 4, 5, 6, and 7.

The *intensities of nutrition* of the leaves of plants growing on the respective plots are plotted in figure 3. These are the sums of the percentages of

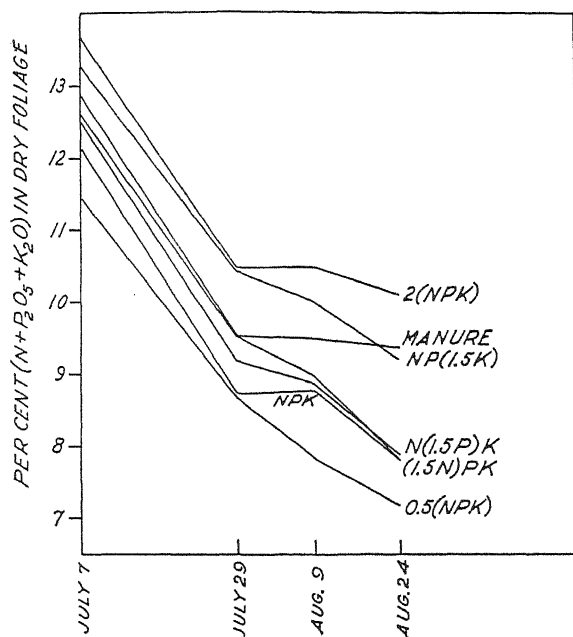


FIG. 3. Intensities of nutrition with advancing age of leaves from plants receiving the different treatments indicated.

( $N + P_2O_5 + K_2O$ ) in the dried foliage at successive dates. The values at any sampling date correspond to the magnitude *s* (column 5, table II).

The mean *NPK-unit* together with the mean *intensities of nutrition* are tabulated in table III together with the absolute and relative yields.

*Relation of foliar diagnosis to yields of tubers.*—These “complete” fertilizer plots can be divided into three groups according to the position of the point representing the mean *NPK-unit* on the triangle (fig. 2).

TABLE III

RELATION OF MEAN NPK-UNIT AND MEAN INTENSITY OF NUTRITION AT FOUR SAMPLING DATES TO YIELDS OF POTATOES ON VARIOUS PLOTS

TREATMENT	TIER	PLOT	MEAN NPK-UNITS (MEAN OF 4 SAMPLINGS)			INTENSITY OF NUTRITION (MEAN OF 4 SAMPLINGS)	YIELDS			
			N	P <sub>2</sub> O <sub>5</sub>	K <sub>2</sub> O		POUNDS PER PLOT	RELATIVE YIELDS	CLASS ORDER	GROUP
Manure .....	1	15	65.00	5.72	29.28	10.33	<i>lb.</i>	%	1 }	A
N(1.5 P)K .....	2	8	65.94	5.47	28.58	9.62	196	100.0	2 }	
NP(1.5 K) .....	2	12	63.68	4.94	31.38	10.74	174	88.7	3 }	B
2(NPK) .....	2	16	63.61	4.78	31.60	11.19	170	86.7	4 }	
NPK .....	1	10	68.65	5.15	26.19	9.39	162	82.7	5 }	C
(1.5 N)PK .....	2	4	69.15	4.99	26.05	9.74	157	80.1	6 }	
0.5(NPK) .....	2	14	68.85	5.44	25.70	8.79	150	76.5	7 }	



*Group A* consisting of the manure plot (no. 1-15) and the N(1.5 P)K plot (no. 2-8) having the highest yields occupying a position on the triangle between groups B and C.

*Group B* consisting of the NP(1.5 K) plot (no. 2-10) and the 2(NPK) plot (no. 2-16) occupying a position on the triangle below and to the left of the manure and N(1.5 P)K plots. The displacement of this group relative to the manure plot is towards the left base apex of the triangle representing  $K_2O = 100$ , and further away from the summit of the triangle representing  $N = 100$ , and from the right base apex representing  $P_2O_5 = 100$ .

*Group C* consisting of the NPK plot (no. 1-10), the (1.5 N)PK plot (no. 2-4) and the 0.5(NPK) plot (no. 2-14) occupying a position on the triangle also to the left of the optimum (manure) plot, but higher up in the triangle. The displacement of this group relative to the optimum (manure) plot is towards the summit representing  $N = 100$ , and farther away from the left base apex representing  $K_2O = 100$ , and from the right base apex representing  $P_2O_5 = 100$ .

*Course of nutrition as indicated by changes in NPK-unit at each successive sampling.*—The displacements, from one sampling date to another, of the *NPK-unit* of the leaves selected from plants growing on the respective plots are given in figures 4, 5, 6, and 7.

Such diagrams show the changes in the equilibrium between nitrogen, phosphoric acid, and potash (NPK). The detailed graphs for all the plots cannot be shown in the same triangle as in figure 3—in which the mean *NPK-units* are shown by a point—because of the intersection of lines representing plots, the *NPK-units* of which are located too near one another.

(a) *General characteristics of the graphs.*—The zigzag nature of these displacements of the *NPK-units* from one sampling date to another are the result of the influence of meteorological factors, and it is of particular importance to note that the influence of these factors on the N- $P_2O_5$ - $K_2O$  equilibrium of a plot varies with the treatment it has received.

The changes in the N- $P_2O_5$ - $K_2O$  equilibrium during the first period (July 7 to 29) result in increasing the nitrogen and phosphoric acid in the *NPK-unit* at the expense of the potash in every treatment. These changes are not equal quantitatively. They are greatest in manure (no. 1-15), less in NPK (no. 1-10), 2(NPK) (no. 2-16), NP(1.5 K) (no. 2-12), and in 0.5(NPK) (no. 2-14), and insignificant in N(1.5 P)K (no. 2-8) and (1.5 N)PK (no. 2-4).

No generalization is apparent from these results. The phenomena described are concurrent with a marked rise in the mean daily temperature and satisfactory conditions with respect to water supply (8, table III)—conditions favorable to an increase in the biological processes in the soil. This period is accompanied by the greatest development of aerial parts of any period.

During the second period (July 29 to August 9) the changes in the  $N-P_2O_5-K_2O$  equilibrium are neither alike qualitatively nor quantitatively. In  $N(1.5 P)K$  (no. 2-8) and also in 2(NPK) (no. 2-16) and NPK (no. 1-10) decreases in the nitrogen and phosphoric acid in the *NPK-unit* take place; whereas in  $(1.5 N)PK$  (no. 2-4) an increase in nitrogen and a decrease in phosphoric acid and potash in the *NPK-unit* occurs, with practically no change in 0.5(NPK) (no. 2-14) and manure (no. 1-15).

Again, no generalization is apparent. The phenomena are concurrent with a drop in mean daily temperature and relatively low precipitation (8, table III).

During the last period (August 9 to 24) the changes in the  $N-P_2O_5-K_2O$  equilibrium are in the direction of a decrease in the nitrogen and phosphoric acid in the *NPK-unit* at the expense of the potash in all treatments. The changes in the *NPK-units* are not quantitatively the same. They are relatively large in manure (no. 1-15), 2(NPK) (no. 2-16), and in  $NP(1.5 K)$  (no. 2-12), and medium in the others. The changes are concurrent with relatively dry conditions and an increase in the mean daily temperature (8, table III).

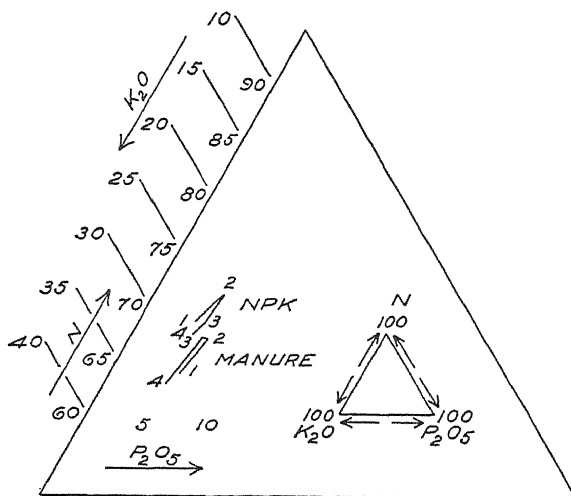


FIG. 4. Course of nutrition as indicated by changes in  $N-P_2O_5-K_2O$  equilibrium (*NPK-units*) with advancing age of leaves from plants growing on manure and NPK plots. Numerals indicate successive sampling periods.

(b) *Course of nutrition in group A—manure (no. 1-15) compared with  $N(1.5 P)K$  (no. 2-8).*—The yield of tubers from the manure plot (no. 1-15) is much higher than from the  $N(1.5 P)K$  plot (no. 2-8). The *NPK-unit* of  $N(1.5 P)K$  (no. 2-8) is located nearer that of manure (no. 1-15)—the optimum plot—than those of any of the other plots. In fact the *NPK-*

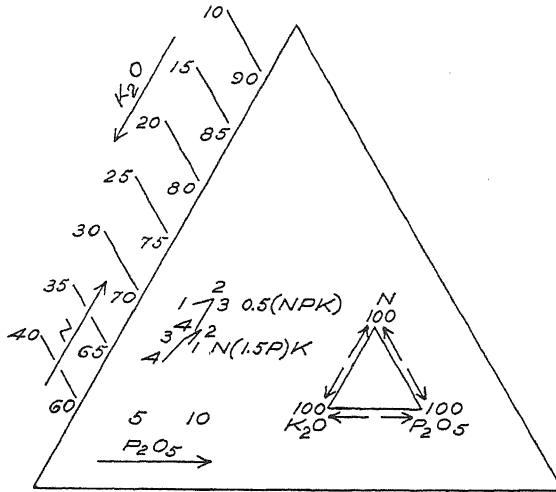


FIG. 5. Course of nutrition as indicated by changes in the  $N-P_2O_5-K_2O$  equilibrium of leaves from plants growing on 0.5(NPK) and  $N(1.5P)K$  plots. Numerals indicate successive sampling periods.

units of (no. 1-15) and (no. 2-8) lie so close together that if this factor be considered alone one would expect the yields to be more nearly equal. The fact that the *intensity of nutrition* of this commercial fertilizer plot is much lower than that of the manure plot is significant, and illustrates the necessity of considering both the *quality* and *quantity* of the mineral nutri-

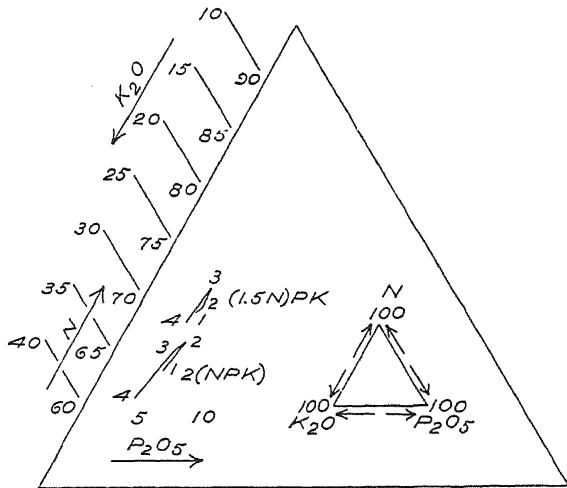


FIG. 6. Course of nutrition as indicated by changes in the  $N-P_2O_5-K_2O$  equilibrium of leaves from plants growing on  $(1.5N)PK$  and  $2(NPK)$  plots. Numerals indicate successive sampling periods.

tion. It will be seen from column 3, table II, that this lowered *intensity of nutrition* is principally due to a too low potash content in the case of plants growing on the N(1.5 P)K plot.

Except at the third sampling the graph for N(1.5 P)K (no. 2-8) is displaced higher on the triangle and slightly to the left in relation to the graph of the optimum (manure) treatment. There is accordingly more N, less  $K_2O$ , and somewhat less  $P_2O_5$  in the *NPK-unit* of N(1.5 P)K (no. 2-8) than in the *NPK-unit* of manure (no. 1-15).

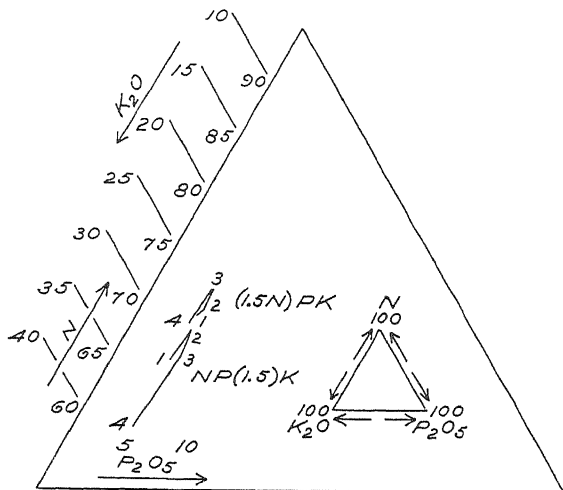


FIG. 7. Course of nutrition as indicated by changes in the N- $P_2O_5$ - $K_2O$  equilibrium (*NPK-units*) with advancing age of leaves from plants growing on (1.5 N)PK and NP(1.5 K) plots. Numerals indicate successive sampling periods.

(c) *Course of nutrition in group B—NP(1.5 K) (no. 2-12) and the 2(NPK) (no. 2-16).*—In comparison with the optimum graph (manure) the *NPK-units* of this group contain with the exception noted less N, less  $P_2O_5$ , and more  $K_2O$ . (During the first period N in the unit of NP(1.5 K) is greater than in manure (no. 1-15).)

(d) *Course of nutrition in group C—(1.5 N)PK (no. 2-4), NPK (no. 1-10), and 0.5(NPK) (no. 2-14).*—The graphs of (1.5 N)PK (no. 2-4) and of 0.5(NPK) (no. 2-14) are displaced a little higher toward the apex  $N=100$  per cent. than that of the NPK (no. 1-10). As already indicated the displacements differ quantitatively. Notable is the 30 per cent. increase in the phosphoric acid in 0.5(NPK) (no. 2-14) during the first period.

(e) *Accommodation of the plant to diverse nutritional equilibrium.*—The yields from the N(1.5 P)K plot (no. 2-8) and the NP(1.5 K) plot (no. 2-12) are relatively close together. However, the *intensities* and the *NPK-units* of the two are very different, which may be interpreted to indi-

cate that the cultivated plant is able to accommodate itself to very diverse nutritional equilibriums with but little (if any) effect on the yield, provided that the various equilibriums are accompanied by a sufficient *intensity of nutrition*.

### Summary

1. The investigations reported earlier (8) have been extended to other plots of the vegetable fertility experiments of the Pennsylvania Agricultural Experiment Station.

2. The plots examined in this paper were treated with rotted horse manure, and also with complete commercial fertilizers differently equilibrated.

3. The indications given by the two methods of graphic representation (8) are described in detail and the foliar diagnosis of all plots compared with that of the optimum (manure) plot.

4. The *intensity of nutrition* (mean) and the composition of the mean *NPK-unit* (8) of the fourth leaf of potato plants growing in the manure plot is 10.35 and 65.0:5.72:29.3, respectively. This intensity and composition of the *NPK-unit* is tentatively regarded as being near the optimum for the potato (variety Rural Russet) in this year.

The writer desires to express his thanks to Dr. WARREN B. MACK for taking the leaf samples.

LABORATORY OF PLANT NUTRITION, DEPARTMENT OF HORTICULTURE  
THE PENNSYLVANIA STATE COLLEGE

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# EFFECTS OF A LIMITING ELEMENT ON THE ABSORPTION OF INDIVIDUAL ELEMENTS AND ON THE ANION:CATION BALANCE IN WHEAT<sup>1</sup>

A. G. MCCALLA AND E. K. WOODFORD

(WITH FIVE FIGURES)

## Introduction

Interrelations of the various elements involved in the absorption of minerals by green plants are among the major problems in plant nutrition. Many types of investigation have been carried out and there is available a vast literature on the effect of one ion on another, the differential absorption of ions in single salt solutions, and the accumulation of individual ions. The general effect on absorption of limiting one element has been given some attention, and during the past few years has been under investigation in this laboratory.

The experiments reported in this paper are concerned with the effects of limiting individual elements on the relative absorption of other elements, and on the general  $\frac{\text{anion}}{\text{cation}}$  balance in the wheat plant.

There are a number of published results dealing with other phases of nutrition which could be recalculated to yield data of the same type as those discussed in this paper. There are some which have a direct bearing on the present discussion, and while no attempt at a complete review has been made, a brief summary of some of these findings is given.

A comprehensive report of the work of LUNDEGÅRDH and his associates is given in a monograph by LUNDEGÅRDH (8). It is impossible to mention many of the individual results, but interrelations of many important nutrients under conditions of limited supply of one or more of them could be calculated from the data presented. Unfortunately, the nitrogen results are not included, so that the calculation of  $\frac{\text{anion}}{\text{cation}}$  ratios is impossible. The interrelations discussed by LUNDEGÅRDH are chiefly those among the cations. Increased absorption of calcium, magnesium, and sodium was observed under conditions of limiting potassium supply, but phosphorus (the only anion studied) was not affected. Little or no consistent variation was noted in the ratio of magnesium to calcium. When nitrate was limited there was no

<sup>1</sup> From the Department of Field Crops, University of Alberta, with financial assistance from the National Research Council of Canada. Paper no. 143 of the Associate Committee on Grain Research of the National Research Council and Dominion Department of Agriculture.

apparent effect on phosphorus, but the potassium content of the plants was much reduced.

The question of absorption and interrelations of nutrients has been further discussed by LUNDEGÅRDII (9). Many of the cation interrelations are discussed, and particular attention is drawn to the antagonisms which may exist, and to the effect of one nutrient in modifying the concentration of another within the plant under various conditions of external concentration.

Interrelations of pairs of elements have received considerable attention at the Research Institute of Plant Physiology of the Imperial College (3). The limitation of chemical analyses to the two elements under investigation restricts the comparison of these with our results; but the effect of gradual reduction of one or both elements on the uptake and distribution of the two is of interest. A discussion of the detailed results is out of the question here. It should be mentioned, however, that GREGORY considers that, at low concentrations, the absorption of an element is largely dependent upon its external concentration.

Many examples of interrelations are to be found in the literature. DIKUSAR (2) reported that when nitrogen was supplied in the form of ammonium there was a depression of the absorption of other cations, the effect being particularly noticeable with potassium. At the same time the absorption of phosphorus was increased. Supplying nitrogen as nitrate increased the absorption of cations and depressed that of phosphorus. The effect of ammonium in depressing the absorption of calcium by tobacco was shown by JACOBSON and SWANBACK (6). It was also demonstrated (14) that when magnesium sulphate was added to a medium previously without magnesium, there was a reduced absorption of potassium and calcium, the latter being forced out into the solution. Many workers have found that phosphate fertilizers increase the phosphorus, and often decrease the nitrogen content of plants. CHAPMAN (1) reported that a nitrogen deficiency caused phosphorus to accumulate in the plant even when the soil was low in phosphorus.

The relations of anions and cations in movement of solutes within the plant are discussed by JACQUES (7). He found a consistently higher equivalent concentration of cations than of anions, but he does not include nitrogen in his calculation of anions.

The uptake of five of the principal elements by lucerne (12) and corn (13) has been studied by RADU. A large number of molecular ratios are presented, but these are all for plants grown under similar conditions. Although the magnitude and trend of some of the ratios are quite different for the two types of plants, there is a marked similarity in the ratio of total anions to total cations. This similarity is also exhibited by results reported by STEPHENSON (15). The mean  $\frac{\text{anion}}{\text{cation}}$  ratio for grasses was much the



same as for legumes despite marked differences in nitrogen and calcium content, and consequently in ratios between pairs of individual nutrients.

### Material and methods

Five series of wheat plants, all but the first having been grown in water cultures, are considered in this paper. The details of production, collection, and analysis of most of the material have been described elsewhere, so only a brief description of each series is given here. Unless otherwise stated, the plants were grown during the spring and summer. Except for series I, where only the above-ground parts were analyzed, results are for the whole plant.

*Series I* was grown under field conditions in 1934. The material designated "complete" in the results was grown at Edmonton on fertile black loam soil, and that designated "limiting nitrogen and sulphur" was grown at Fallis, 50 miles west of Edmonton, on relatively infertile gray podsolie loam. Analyses were made on eight individual replicates of each of two varieties of wheat grown on the two soils. Details are given by WOODFORD and MCCALLA (17). The values given in this paper are means for the two varieties.

*Series II* was grown in water cultures in 1936, and included "complete" and "limiting nitrogen" ( $\frac{1}{2}$  complete) solutions. All solutions were made up in distilled water, and adequate precaution was taken to insure against deficiency of any element other than nitrogen. Limited nitrogen was compensated for by equivalent increases in phosphorus. Analyses were made on duplicate samples at five stages of development. Details are given in another paper (11).

*Series III* was a supplementary series grown in water cultures for 35 days in the fall of 1936. All material received a complete solution for the first six days, after which the six solutions used were as follows: nitrogen-free; limiting nitrogen at  $\frac{1}{2}$  and  $\frac{1}{4}$  of the complete; complete; limiting phosphorus at  $\frac{1}{4}$  of the complete; and phosphorus-free. Limited nitrogen or phosphorus was compensated for by equivalent increases in the other nutrient. There were no other changes in the solutions. At the end of the 35 days the whole crop was harvested and analysed.

*Series IV* was grown in water cultures in 1934, and included "complete" and "limiting potassium" ( $\frac{1}{4}$  of complete) solutions. Although several different conditions of limitation were employed, only that in which limited potassium was compensated for by an equivalent increase in calcium is here considered. Tap water, containing a small amount of sodium, was used in making up these solutions. The first seeding of this series, on which analyses were made at three stages, was unfortunately lost as a result of an accident in the greenhouse when the plants were 44 days old. A second seed-

ing was made, and the plants analyzed at 31 and 54 days of age. The results for the two seedlings are recorded separately. Details regarding this series are given in an earlier paper (10).

*Series V* was grown in water cultures in 1936 and included "complete" and "limited calcium" ( $\frac{1}{10}$  of complete) solutions. The reduction in calcium was compensated by an equivalent increase in potassium. Duplicate analyses were made at five stages of maturity. The same general procedure was used in production, and the same methods of analyses as for series II.

For all the material grown in water culture, the complete solution used was Hoagland's complete nutrient solution made up as follows: 5 cc. of molar  $\text{Ca}(\text{NO}_3)_2$ , 5 cc. of molar  $\text{KNO}_3$ , 2 cc. of molar  $\text{MgSO}_4$  and 1 cc. of molar  $\text{KH}_2\text{PO}_4$  per litre of solution. Ferric tartrate at the rate of 1 cc. of 0.5 per cent. per litre was added every third or second day during the experiment.

## Results

Analytical results from these studies are here confined to a minimum. Most of the detailed results have been published elsewhere (10, 11, 17), and for the purpose of the present discussion are unnecessary.

In calculating the ratios, two assumptions had to be made. The first was that nitrogen is absorbed as an anion, and, except for the first experiment, this is the only justifiable assumption, since all nitrogen was supplied as nitrate. While this is not proof that it is absorbed as such, it seems certain that nitrate was absorbed in large quantities, because it formed an appreciable proportion of the total nitrogen of the complete culture plants at all stages, and over 50 per cent. of the nitrogen in the leaves and stems at maturity. The field-grown material of series I also contained small amounts of nitrate, and since the various ratios in field- and culture-grown material were much alike, it was assumed the nitrogen was absorbed as nitrate. The second assumption was that phosphorus is absorbed as a divalent anion, an assumption which is possibly not as sound as the first. The reason for this assumption was that in all earlier culture experiments the pH of the solutions very rapidly reached and was maintained at approximately 7.2. At this pH more phosphate is in the form of  $\text{HPO}_4^-$  than in the form of  $\text{H}_2\text{PO}_4^-$ . An accurate allowance for the two forms was impossible, since there were differences in pH from time to time and from culture to culture. If it had been assumed that the absorption of phosphorus took place as the univalent  $\text{H}_2\text{PO}_4^-$  the results and conclusions would have been only slightly affected by this change in procedure. In only one specific instance (series III) was a marked difference in the pH of different solutions found, and this is discussed in its place.

The term "limiting" is used in this paper because the effects of moderate limitation of an element are much more striking than those of a mod-

erate excess. In series II, for example, the solution termed "limiting nitrogen" might have been designated "excess phosphorus." Similarly in the other water culture series, the solutions might have been designated by the element in excess rather than by that in limitation. This is not true of series I because there was no excess phosphorus in the podsol; in fact this element was definitely lower than in the black loam.

All ratios presented in the following sections are on a chemically equivalent basis.

#### LIMITING ANIONS

##### *Series I. Limiting nitrogen and sulphur under field conditions*

A summary of the analytical results for wheat grown under conditions of complete (black loam soil), and limiting nitrogen and sulphur (podsolic loam) nutrition is presented in table I. The ratios between individual pairs and between various combinations of nutrients are presented in figure 1. These results are for the above-ground parts only of the plant.

TABLE I  
NUTRIENT CONTENT AS PERCENTAGE DRY MATTER OF WHEAT  
SERIES I

NUTRIENT	SOIL NUTRIENTS	NUTRIENT CONTENT				
		TIME FROM SEEDING (DAYS)				
		29	48	64	90	115
		%	%	%	%	%
Nitrogen .....	Complete* .....	5.62	3.96	2.24	1.40	1.46
" .....	Limiting N and S† .....	5.23	1.88	1.08	0.86	0.94
Sulphur (SO <sub>4</sub> ) .....	Complete .....	1.30	1.00	0.73	0.64	0.42
" .....	Limiting N and S .....	1.10	0.44	0.31	0.30	0.30
Phosphorus (P <sub>2</sub> O <sub>5</sub> ) .....	Complete .....	0.93	0.60	0.37	0.28	0.24
" .....	Limiting N and S .....	1.23	0.82	0.52	0.46	0.48
Potassium .....	Complete .....	4.38	4.08	2.88	1.16	0.78
" .....	Limiting N and S .....	3.69	2.72	1.92	0.97	0.70
Calcium .....	Complete .....	0.90	0.60	0.34	0.25	0.20
" .....	Limiting N and S .....	0.72	0.35	0.20	0.13	0.16
Magnesium .....	Complete .....	0.40	0.23	.....	0.06	0.08
" .....	Limiting N and S .....	0.28	0.11	.....	0.06	0.07

\* Fertile black loam soil.

† Relatively infertile gray podsolic loam.

The most striking feature of the results is the low nitrogen and sulphur content and the correspondingly higher phosphorus content of the wheat grown on the podsol. All cations were lower in the plants with limited nitrogen and sulphur, but there was no indication that any of these, except possibly calcium to a slight extent, was limiting development. This is more clearly seen from the ratios in figure 1. The limited uptake of nitrogen

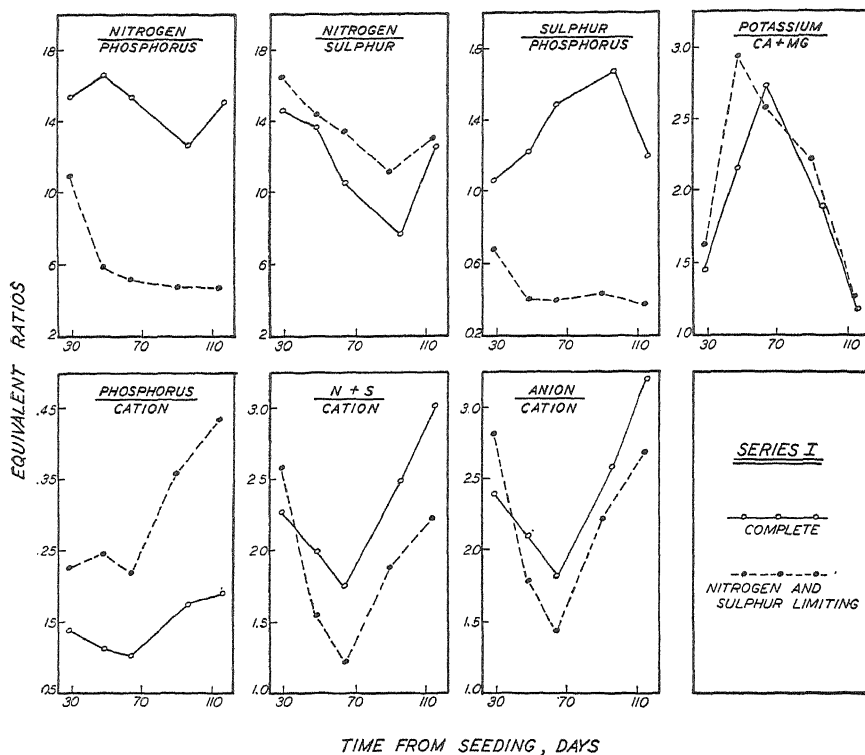


FIG. 1. The effects of limiting nitrogen and sulphur on various equivalent ratios, series I.

and sulphur in relation to phosphorus is clearly evident in these graphs. It is evident also that sulphur was slightly more limiting than nitrogen.

The ratios involving cations show that there was no marked difference in the proportion of potassium, calcium, and magnesium taken up from the two soils. Thus, although all cations were lower in the plants grown on the podsol, this lower level was apparently more closely related to the reduction of total anion absorption than to any relative deficiency in cation supply.

Despite the relatively large differences in nitrogen, sulphur, and phosphorus, the total  $\frac{\text{anion}}{\text{cation}}$  balance in the plants grown on the podsol was fairly well maintained both by the increase in phosphorus and the decrease in cation absorption. It must be stressed here that although only percentage results are presented in table I, the ratios are not affected by differences in general level of yield. Therefore, while the total amount of phosphorus absorbed by the plants growing on the podsol was actually lower than that absorbed by the others, the relative absorption was much greater. Similarly, the total weight results (17) show that the actual cation absorption

from the podsol was only about one-fourth that from the black loam, but the relative cation absorption, while less, was not nearly so much less as the weight figures might suggest at first glance. The  $\frac{\text{anion}}{\text{cation}}$  balance has been briefly discussed by WOODFORD and MCCALLA (17). The general effect of limiting nutrients on this balance is better discussed after the presentation of the results from the other series.

*Series II. Limiting nitrogen under culture conditions*

A summary of the results for series II is presented in table II, and the various equivalent ratios in figure 2.

TABLE II  
NUTRIENT CONTENT AS PERCENTAGE DRY MATTER OF WHEAT  
SERIES II

NUTRIENT	SOLUTION	NUTRIENT CONTENT				
		TIME FROM SEEDING (DAYS)				
		29	42	54	66	92
		%	%	%	%	%
Nitrogen .....	{ Complete .....	4.88	3.70	2.39	2.03	1.95
	{ Limiting N .....	3.72	2.37	1.57	1.27	1.01
Phosphorus (P <sub>2</sub> O <sub>5</sub> ) .....	{ Complete .....	2.92	2.34	1.83	1.36	1.57
	{ Limiting N .....	3.13	2.61	2.09	2.09	2.71
Sulphur (SO <sub>4</sub> ) .....	{ Complete .....	1.20	1.00	0.73	0.60	0.88
	{ Limiting N .....	1.48	1.50	1.23	1.11	1.24
Potassium .....	{ Complete .....	7.20	5.40	3.41	2.77	2.43
	{ Limiting N .....	6.53	4.56	2.94	2.43	1.57
Calcium .....	{ Complete .....	0.66	0.52	0.38	0.49	0.45
	{ Limiting N .....	0.54	0.40	0.27	0.28	0.30
Magnesium .....	{ Complete .....	0.15	0.23	0.18	0.16	0.20
	{ Limiting N .....	0.12	0.12	0.08	0.11	0.14

Before discussing the ratios, attention must be drawn to the much higher phosphorus values for this series than for series I, and the consequent lower  $\frac{\text{nitrogen}}{\text{phosphorus}}$  and  $\frac{\text{sulphur}}{\text{phosphorus}}$  ratios. The most likely explanation would seem to be that increased phosphorus uptake resulted from the exclusion of other anions when the solutions were made up in distilled water. It has been reported (14) that when chloride is absorbed, the phosphorus content of the plant decreases, and it seems likely that, with the virtual exclusion of anions except nitrate, sulphate, and phosphate from the solution, the increased phosphorus absorption compensated for other anions usually absorbed.

The form and trend of the curves for  $\frac{\text{nitrogen}}{\text{phosphorus}}$  ratios are the same as

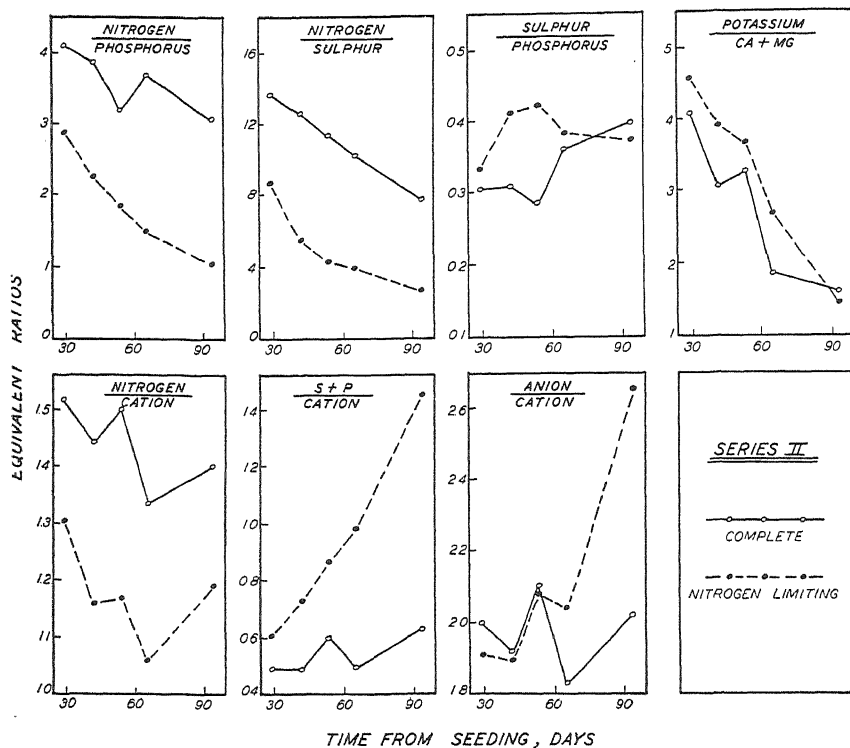


FIG. 2. The effects of limiting nitrogen on various equivalent ratios, series II.

or series I, but the  $\frac{\text{nitrogen}}{\text{sulphur}}$  and  $\frac{\text{sulphur}}{\text{phosphorus}}$  ratios are, of course, quite different, since sulphur was not limiting in this series. Limiting nitrogen had a negligible effect on the  $\frac{\text{sulphur}}{\text{phosphorus}}$  ratio, as the percentage results in table II show that limiting nitrogen increased the relative uptake of both phosphorus and sulphur. There was little effect on the ratios of cation to anion, so that the positions of the curves for the two treatments in the  $\frac{\text{nitrogen}}{\text{cation}}$  and  $\frac{\text{S + P}}{\text{cation}}$  graphs are determined by the anion absorption.

The  $\frac{\text{anion}}{\text{cation}}$  balance was but slightly affected by the nutrition during the first half of the growth period. The sharp upward swing of the curve for limited nitrogen plants can be discounted considerably, because a large proportion of the marked phosphorus increase (table II) was in the roots only, and there is good reason to believe that probably most of this increase was due to phosphorus deposited on the surfaces of the roots. These roots yielded ash solutions which were brown with iron salts, but most of this

iron had apparently not been absorbed, and was probably deposited on the surface as iron phosphate. Had the importance of this effect been realized at the time of analysis, allowance could probably have been made for it.

The fact that the  $\frac{\text{anion}}{\text{cation}}$  ratio in the mature limited-nitrogen plants is high, instead of low as in series I, adds evidence in favor of the idea that not all of the phosphorus was absorbed.

*Series III. Limiting nitrogen and phosphorus under culture conditions*

The results for series II appeared so definite that it was decided to check them by using a graded series of nitrogen and phosphorus concentrations. Owing to the short period of time during which good light conditions were available in the fall, and to the limitation of space for different treatments, these plants were grown only for 35 days. The analytical results are presented in table III and the various ratios in table IV. The compensating

TABLE III  
NUTRIENT CONTENT AS PERCENTAGE DRY MATTER OF WHEAT  
SERIES III, ALL 35 DAYS FROM SEEDING

NUTRIENT	NUTRIENT CONTENT					
	SOLUTION					
	N-FREE (AFTER 6 DAYS)	LIMITING N ( $\frac{1}{40}$ COM- PLETE)	LIMITING N ( $\frac{2}{10}$ COM- PLETE)	COMPLETE	LIMITING P ( $\frac{1}{40}$ COM- PLETE)	P-FREE (AFTER 6 DAYS)
	%	%	%	%	%	%
Nitrogen .....	2.50	3.18	3.47	3.91	3.74	3.77
Phosphorus (P <sub>2</sub> O <sub>5</sub> ) .....	6.61	5.99	4.32	3.38	0.62	0.51
Sulphur (SO <sub>4</sub> ) .....	2.40	1.68	2.25	1.56	1.20	1.17
Potassium .....	5.38	5.24	5.79	5.69	4.87	3.95
Calcium .....	.....	0.19	0.26	0.54	0.28	0.28
Magnesium .....	0.30	0.17	0.29	0.33	0.24	0.29

effect of phosphorus under low nitrogen conditions is definitely borne out by these results. Under limiting phosphorus nutrition the reverse effect was not observed, but there was a decided depression (due in large part to potassium) in the amount of total cation absorption.

The  $\frac{\text{anion}}{\text{cation}}$  ratios were fairly constant; only under the two most limiting nitrogen conditions were they definitely higher than for the complete. In these solutions, however, the pH values were decidedly lower than that of the complete, the difference being as great as from 5 to 7.2. This difference may have had an effect on the form in which phosphorus was absorbed, and

TABLE IV  
EQUIVALENT RATIOS  
SERIES III

RATIO	NUTRIENT CONTENT					
	SOLUTION					
	N-FREE (AFTER 6 DAYS)	LIMITING N ( $\frac{1}{4}$ %) COM- PLETE)	LIMITING N ( $\frac{1}{2}$ %) COM- PLETE)	COMPLETE	LIMITING P ( $\frac{1}{4}$ %) COM- PLETE)	P-FREE (AFTER 6 DAYS)
N/P .....	0.96	1.34	2.04	2.93	15.5	18.4
N/S .....	3.57	6.47	5.27	8.65	10.8	11.2
S/P .....	0.27	0.21	0.39	0.34	1.43	1.65
N/cation .....	1.07	1.44	1.34	1.39	1.68	1.94
S/cation .....	0.30	0.22	0.25	0.16	0.16	0.17
P/cation .....	1.12	1.07	0.66	0.47	0.11	0.11
K/Ca + Mg .....	3.50	5.52	3.96	2.68	3.70	2.65
Anion/cation .....	2.49	2.73	2.25	2.03	1.95	2.21

a recalculation of the  $\frac{\text{anion}}{\text{cation}}$  ratios, assuming phosphorus to be absorbed as  $\text{H}_2\text{PO}_4^-$ , makes the levels for the various treatments more nearly uniform, the values for the complete and N-free plants being 1.79 and 1.93.

Both the compensating effect of phosphorus and the generally uniform anion-cation balance are illustrated by the bar diagrams in figure 3. Equivalent percentages, rather than equivalents absorbed, are plotted in

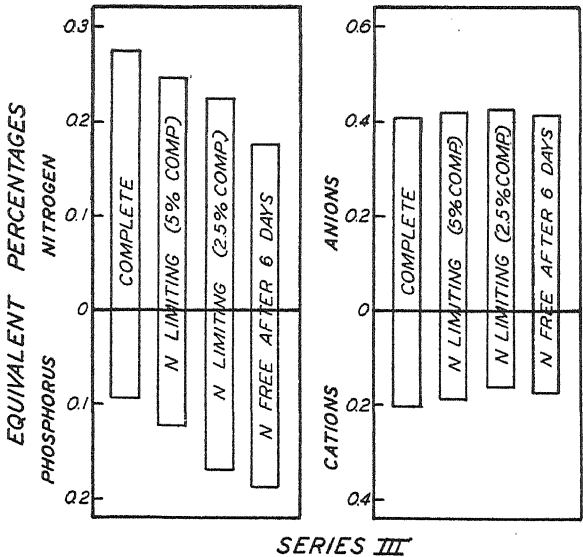


FIG. 3. The effect of limiting nitrogen on the  $\frac{\text{P}}{\text{N}}$  and  $\frac{\text{anion}}{\text{cation}}$  ratios, series III.



order to eliminate the differences in level of yield from the various treatments. The relative relationship would be unaltered by plotting weights instead of percentages.

The absorption of nitrogen and phosphorus was roughly parallel to the concentration supplied in the culture solution, especially in the limiting nitrogen cultures. Calculations of the actual milligrams of nitrogen and phosphorus absorbed shows that under no treatment was the absorption of the limited element reduced to a level corresponding with the reduction in concentration supplied; nor was the absorption of the element in excess increased to a level corresponding with the increase in concentration. Furthermore, while the concentration of nitrogen in the low phosphorus solutions was higher than in the complete, the absorption was definitely less. In the plants grown in these solutions the ion balance was maintained by decreased cation absorption, so concentration could have played little part.

#### LIMITING CATIONS

##### *Series IV. Limiting potassium under culture conditions*

A summary of the analyses of plants in series IV is presented in table V, and the equivalent ratios in figure 4. These results cover the first 54 days

TABLE V  
NUTRIENT CONTENT AS PERCENTAGE DRY MATTER OF WHEAT  
SERIES IV

NUTRIENT	SOLUTION	NUTRIENT CONTENT				
		TIME FROM SEEDING (DAYS)				
		FIRST SEEDING			SECOND SEEDING	
		20	31	44	31	54
		%	%	%	%	%
Nitrogen .....	{ Complete .....	5.52	5.43	4.98	4.30	3.54
	{ Limiting K ...	4.93	4.76	3.92	3.82	2.87
Phosphorus (P <sub>2</sub> O <sub>5</sub> )...	{ Complete .....	3.93	2.86	2.39	1.53	1.03
	{ Limiting K ...	3.29	2.51	2.38	1.58	1.10
Potassium .....	{ Complete .....	6.38	6.88	7.06	6.13	5.68
	{ Limiting K ...	2.14	1.99	1.49	1.80	1.80
Calcium .....	{ Complete .....	0.58	0.72	0.74	0.70	0.57
	{ Limiting K ...	0.94	1.14	1.30	1.13	1.14
Magnesium .....	{ Complete .....	0.36	0.32	0.32	0.30	0.30
	{ Limiting K ...	0.54	0.49	0.53	0.41	0.46

of development only, and are for two sets of plants as described in the section on material. In general, the percentage results were higher for the 31-day-old plants of the first set than for those of the second, but the ratios

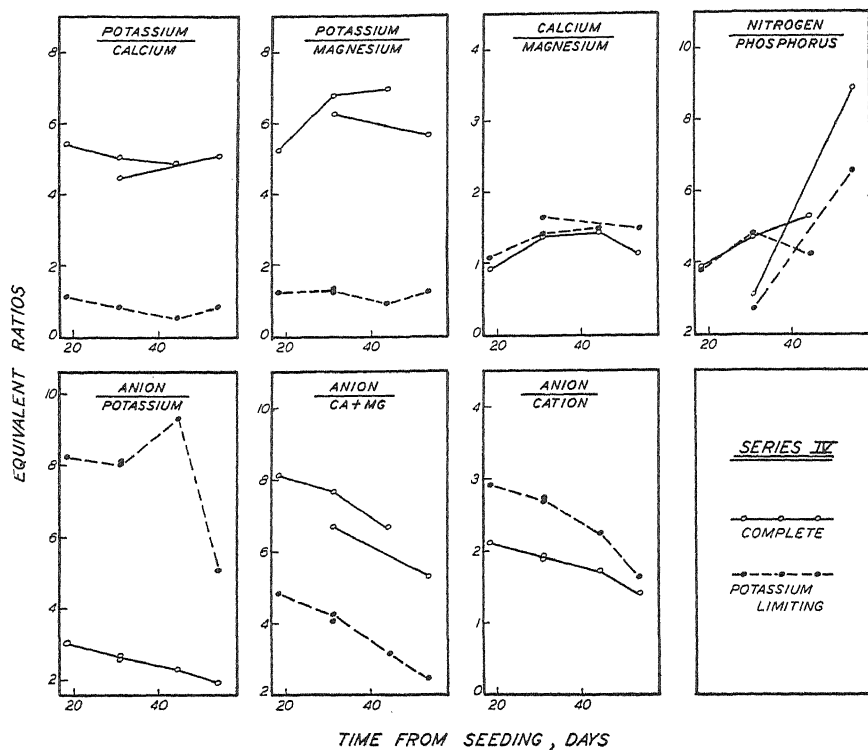


FIG. 4. The effects of limiting potassium on various equivalent ratios, series IV.

are, for the most part, in excellent agreement, and indicate that the error of culture technique was relatively low.

The limitation of potassium resulted in an increased relative absorption of both calcium and magnesium, but the proportions of the two divalent cations was unaffected. These results are in agreement with those already cited (14) and with those of HOAGLAND (4), who points out that very few cases of interrelations between potassium and magnesium have been reported.

While the  $\frac{\text{nitrogen}}{\text{phosphorus}}$  ratios were irregular over the period of the experiment, there was little if any significant difference as a result of potassium limitation. In this series, as in series II and III, the  $\frac{\text{nitrogen}}{\text{phosphorus}}$  ratios were much lower than those found under field conditions. While tap water was used in making up these solutions, the only anion whose concentration was appreciably increased by this procedure was sulphate, which was constant for the different solutions.

The relations between anions and potassium, anions and calcium and

magnesium, and anions and total cations again emphasize the tendency for the  $\frac{\text{anion}}{\text{cation}}$  balance in plants growing under restriction of one nutrient to be maintained by increased absorption of other ions of the same sign. The difference between the two curves for  $\frac{\text{anion}}{\text{cation}}$  ratios might have been much less had sodium been determined. Sodium was present in the tap water, and although it appears that the normal, completely nourished wheat plant absorbs comparatively little sodium (5, 8), one limited as to potassium supply may decidedly increase this absorption (8).

*Series V. Limiting calcium under culture conditions*

A summary of the analytical results for series V is presented in table VI, and the equivalent ratios are given in figure 5.

TABLE VI  
NUTRIENT CONTENT AS PERCENTAGE DRY MATTER OF WHEAT  
SERIES V

NUTRIENT	SOLUTION	NUTRIENT CONTENT				
		TIME FROM SEEDING (DAYS)				
		29	42	54	66	92
		%	%	%	%	%
Nitrogen .....	{ Complete .....	4.88	3.70	2.39	2.03	1.95
	{ Limiting Ca ...	4.62	2.98	2.35	2.01	1.78
Phosphorus ( $P_2O_5$ ) ...	{ Complete .....	2.92	2.34	1.83	1.36	1.57
	{ Limiting Ca ...	2.68	2.19	1.61	1.79	1.47
Sulphur ( $SO_4$ ) .....	{ Complete .....	1.20	1.00	0.73	0.60	0.88
	{ Limiting Ca ...	1.14	0.80	0.77	0.74	1.07
Potassium .....	{ Complete .....	7.20	5.40	3.41	2.77	2.43
	{ Limiting Ca ...	5.29	4.15	3.30	3.13	2.39
Calcium .....	{ Complete .....	0.66	0.52	0.38	0.49	0.45
	{ Limiting Ca ...	0.31	0.27	0.15	0.18	0.17
Magnesium .....	{ Complete .....	0.15	.....	0.18	0.16	0.20
	{ Limiting Ca ...	0.18	.....	0.21	0.22	0.38

The limitation of calcium resulted in increased relative absorption of both potassium and magnesium, with the increase in magnesium considerably the greater. Sodium did not enter the cation balance of this series because distilled water was used in all solutions.

The limitation had no effect on the  $\frac{\text{nitrogen}}{\text{phosphorus}}$  ratio. The  $\frac{\text{anion}}{\text{calcium}}$  ratios were much higher for the plants limited as to calcium than for the complete,

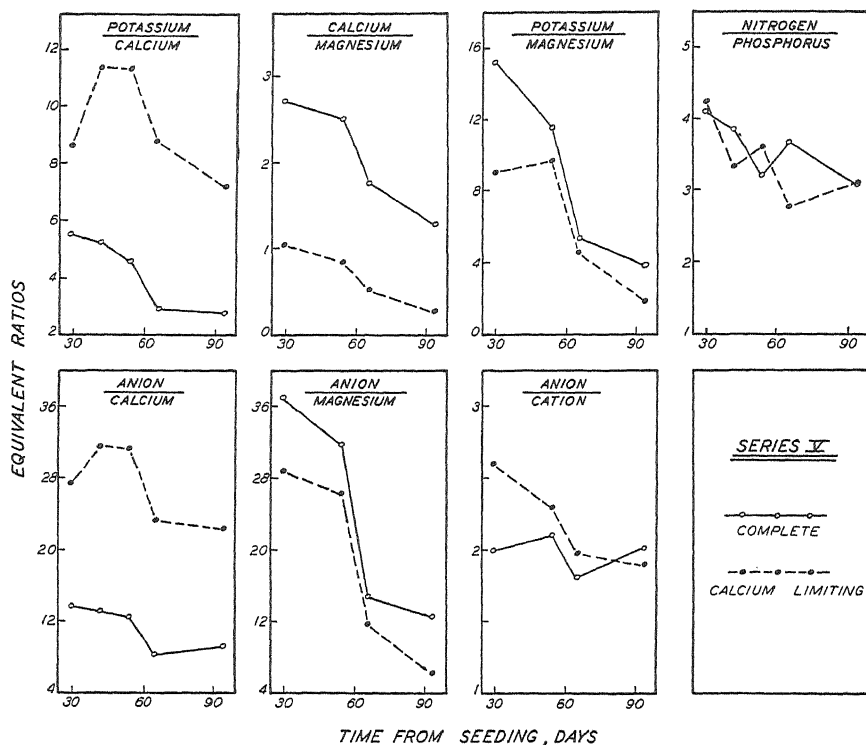


FIG. 5. The effects of limiting calcium on various equivalent ratios, series V.

but the compensating effect of magnesium and potassium reduced the differences in  $\frac{\text{anion}}{\text{cation}}$  ratios to a relatively low level. The general effects of limitation were, therefore, of the same nature as in all other series.

### Discussion

Most of the material used in this study was produced for purposes other than those discussed, and while the experiments reported in this paper deal with absorption and interrelations of various nutrients, it is recognized that they have little in common with the precise, short-period absorption experiments carried out by STEWARD (16) and others. There is here no possibility of dealing with absorption as distinct from growth. Obviously, most of the nitrogen, a large part of the phosphorus and sulphur, and some part, at least, of the cations determined, are in metabolized form. The results as here reported give the total absorption over definite periods, and as such reflect the interrelations of the various nutrients regardless of the exact mode of absorption, or the external and internal factors affecting this

process. The nature of our experiments makes any application of the results to the mechanism of absorption unwarranted.

Under conditions of growth which were similar, except for the mineral nutrition, there was a consistent tendency toward a maintained balance between anion and cation absorption. In all except the field-grown series I, the plants receiving different treatments were grown together under the same conditions of light, temperature, etc.

The ion balance was always decidedly in favor of the anions, regardless of the nutrition of the plant. This balance is directly the opposite of that discussed by JACQUES (7), and the difference is, of course, accounted for by our inclusion of nitrate in calculating the ratios. In this work the interest is in the uptake of nutrients from the soil, while JACQUES discusses the movement from cell to cell within the plant. While a complete discussion of this question is out of order here, it must be stated that we cannot accept the reasoning of JACQUES, particularly if this is extended to the absorption of nutrients from the soil. Nitrogen cannot be neglected in absorption, nor can absorption from the soil be assumed to take place as equal amounts of  $\text{NO}_3^-$  and  $\text{NH}_4^+$ . It is sufficient to say, perhaps, that it is difficult to reconcile the presence of nitrogen-containing organic acids and the coincident movement of a strong hydroxide at the pH's which JACQUES regards as obtaining in the cells under discussion. The argument that nitrogen is present in higher equivalent concentration than the total cations is in agreement with most of our results; but ion exchange, which is casually dismissed by JACQUES, may account for the preponderance of anions over cations. As already mentioned, our results do not permit the drawing of any conclusions regarding mode of absorption, but in any case, the reasoning of JACQUES as applied to absorption by higher plants does not make adequate provision for the uptake of nitrogen.

The other definite result obtained is the effect of a limiting nutrient in increasing absorption of another ion of the same sign or in decreasing the total absorption of ions of the opposite sign. If nutrients were absorbed independently, or if cations had no effect on anions, there seems to be no good reason why the limitation of nitrogen, for example, should be so consistently accompanied by increased absorption of phosphorus or decreased cation absorption. The nearly equivalent compensation by phosphorus of the limited nitrogen uptake in series III indicates that phosphorus absorption was increased to maintain a fairly definite  $\frac{\text{anion}}{\text{cation}}$  balance. In the limiting phosphorus cultures of series III the balance was maintained by a decrease in cation absorption, while in series I both of the factors were operative.

Another possible explanation of some of our results, particularly those

of the limited nitrogen cultures in series III, is that the absorption of an element by the plant depends on the external concentration of the element. Thus high phosphorus absorption by plants grown in limited nitrogen solutions would be the result of increased concentration of phosphorus rather than of decreased absorption of nitrogen. Certainly where concentrations are low, there is a direct relation between absorption over short periods and external concentration (3). This explanation of the results cannot be entirely dismissed, since in many of our experiments it is impossible to separate the two effects, but in many other instances of apparent compensation, concentration effects must have been very secondary. A brief consideration of a few such cases may be in order.

The higher phosphorus absorption by the plants grown on the podsol (series I) was in no way related to concentration, because both total and available phosphorus were lower in the podsol than in the fertile black loam from which the plants removed relatively much less phosphorus.

The fact that more phosphorus was absorbed from solutions made up in distilled water than from soil, could scarcely be a concentration effect since the  $\frac{\text{nitrogen}}{\text{phosphorus}}$  ratio in the solutions was much higher than in the soil. Similarly the increased phosphorus absorption by plants as a result of nitrogen limitation in series II was not proportionately greater than the increase in sulphur absorption, despite the fact that the external phosphorus concentration had been increased to more than eight times that of the complete solution, while the sulphur concentration was unchanged.

The absorption of calcium and magnesium was increased by limitation of potassium in series IV, the increases being proportionately the same, although only calcium concentration was increased in the solution. Magnesium absorption was increased more than that of potassium when calcium was limited in series V, although only potassium concentration was increased.

In all of these experiments, the concentration of any element in the complete solution was probably sufficient to give maximum absorption, and therefore increases in absorption as a result of increased concentration in the solution would not necessarily be expected. The increased absorption under these conditions, the fact that the increase was not confined to the element in excess, and the fairly constant  $\frac{\text{anion}}{\text{cation}}$  balance are all evidence in favor of the compensation rather than the concentration effect.

In all series involving a limitation of nitrogen there appears to be an accompanying decrease in calcium absorption. In series IV, however, the relatively enormous increase in calcium absorption is accompanied by a somewhat decreased nitrogen uptake. In series V, the limitation of cal-

cium had little effect on nitrogen uptake, so the apparent relationship between the two nutrients, as indicated by series I to III, seems open to question. In series I and II, in which nitrogen uptake was limited, the decrease in calcium was only slightly greater than that in potassium absorption; while in series III the uptake of calcium was retarded by limitation of both nitrogen and phosphorus. Apparently only by studying the effect of the two elements at the same time can reliable conclusions regarding interrelations be obtained, and some such system as that used by GREGORY and his students (3) appears to offer the best means of studying specific pairs of elements. Such systems would yield excellent material for studying the effect of limiting nutrients on the total  $\frac{\text{anion}}{\text{cation}}$  balance providing analyses were extended to include all of the principal elements involved in absorption. Such studies are now being carried out in this laboratory. Because all the apparent interrelations between pairs of elements not definitely under investigation in this work are subject to the same limitation as those between nitrogen and calcium, no others are considered.

### Summary

1. Limiting the supply of one nutrient resulted in an increased uptake of another nutrient absorbed as an ion of the same sign, or in a decreased total uptake of the nutrients absorbed as ions of the opposite sign.

2. When nitrogen was limiting, the effect was chiefly felt in increased phosphorus absorption, although there was also increased sulphur absorption in some series. In one particular series of graded nitrogen and phosphorus supply, the increased phosphorus absorption resulting from limiting nitrogen supply was fully equivalent to the decreased nitrogen uptake.

3. Limiting potassium was accompanied by increased absorption of both calcium and magnesium. Limiting calcium was accompanied by relatively increased absorption of potassium and magnesium and slightly decreased absorption of anions.

4. Despite relatively enormous differences in ratios of individual nutrients as a result of limiting one, there was a marked tendency toward a maintained balance between total anions and cations. This balance was always in favor of the anions.

DEPARTMENT OF FIELD CROPS

UNIVERSITY OF ALBERTA, EDMONTON, CANADA

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# RELATION BETWEEN LIGHT AND THE ELECTRIC POLARITY OF *CHARA*

SIDNEY O. BROWN

(WITH SEVEN FIGURES)

## Introduction

The purpose of this investigation was to study the nature of the effect of light on the electric polarity of *Chara* and to determine the possible relation of the electric polarity to photosynthesis. As a basis for the final experiments, the normal distribution, magnitude, the degree of stability of the electrical potentials, and the effect of mechanical stimulation were first determined.

An electrical polarity corresponding to the morphological polarity in the axis has been established and studied in detail in *Obelia* and other hydroids (2, 9), the Douglas fir (14), the onion root, (11, 19), frog skin (3, 15), *Avena* coleoptile (5, WILKS, in press), *Phaseolus vulgaris* (27) and other polar structures. These continuously maintained E. M. F.'s have been shown to be quantitatively linked with the oxidation mechanisms of the living cell (12, 25, 26). The E. M. F. appears to depend upon the flux equilibrium in an electrochemical system made up of substances necessary for cell oxidation, and in the presence of atmospheric oxygen (10). The polarities of these living systems have been shown to be affected by changes in temperature, anaesthetics, oxygen, mechanical stimulation, light, externally applied E. M. F.'s, and other factors, which, directly or indirectly, affect cell oxidation. From the numerous facts obtained from this work LUND (12) has formulated a theory of cell correlation which attempts to explain the method by which a group of electrically dominant cells may control the rate and orientation of physicochemical processes in adjacent cells. Some of the new facts presented in this paper appear to have a bearing on this problem.

Previous investigations on the electrophysiology of *Chara* are those by JOST (8), who determined the effect of various electrolytes on the potential, and by AUGER (1), who reported that stimulation resulted in a negative wave of excitation which moved along the axis at the rate of 6 cm. per second. OSTERHOUT and his co-workers (22, 23, 26) have made extensive investigations on the conditions of stimulation and the transmission of the excitatory wave in *Nitella*, a close relative of *Chara*. As far as the writer is aware, the effect of light on the electrical potential of neither *Chara* nor *Nitella* has ever been investigated.



*vulgaris*. The internodes consist of long multinucleate cells covered by a cortical layer of much smaller spirally arranged cells. At the node, 7 to 10 leaves form a whorl; each leaf has, in general, the same structure as the axis. In figure 1 B the region designated as the apex consists of three or four of the most apical unelongated internodes, covered by leaves of the lower whorls. For experimental purposes the "apex" is, therefore, defined here as all apical structures above the first internode, which is at least 10 millimeters in length. The total axial length of the apex is not more than three millimeters. In figure 1 B the electrode positions along the main axis of the plant are designated by letter. The average length of the first internode was 11 mm., of the second 17 mm., and of the third 20 mm. The part of the "standard preparation" used in the investigation of the effect of light and mechanical stimulation, unless otherwise specified, consisted of the apex, the first internode and node, or that part of the plant between A and D in figure 1 B. If microscopic examination after each experiment showed injury, or if the plant failed to grow on its return to the aquarium, the data obtained from such material were discarded.

An unsuccessful preliminary attempt to determine the electric polarity of the axis of *Chara* indicated that an apparatus had to be constructed which would enable measurements to be made in an atmosphere saturated with water vapor under relatively constant condition of light and temperature with a minimum of mechanical disturbance. Figure 1 A shows the apparatus which was finally constructed and which fulfills these requirements. It consists of a rack and pinion, R, on which is mounted the base, N, supporting a glass aquarium with the inside dimensions of  $16 \times 10 \times 8$  cm. A bakelite cover with a large window, W, two small and three large perforations is sealed with a vapor-tight joint to the top of the aquarium. Opaque fingerstalls, Z, form flexible diaphragms over the large openings, to prevent the loss of moisture.

One end of a bent glass rod, S, is attached to a micromanipulator (not shown in the figure). The other end of the glass rod passes through the middle opening and forms a support to which is cemented a tube serving as a receptacle, Q, for a plant holder, H. This receptacle receives a small glass rod cemented to two glass hooks. One of these hooks fits below the first node and the other below the second and serves as a support for the preparation.

The electrode contacts are made (1) by the meniscus at the surface of the water in the aquarium and (2) by a tube, K', filled with water which connects to the usual small claw contact just below the apex of the plant. The water used in the electrode contacts and in the aquarium is the medium in which the plant was growing. Electrodes  $M_1$  and  $M_2$  of isoelectric zinc amalgam, saturated zinc sulphate type, are connected by leads,  $L_1$  and  $L_2$ , to a previously calibrated Compton electrometer. To prevent injury to the

plant by the diffusion of zinc sulphate from the electrodes the contacts were back washed without mechanical disturbance to the plant before each reading by allowing some water to run out through the stopcocks,  $U_1$  and  $U_2$ , shown in the figure. The water vapor saturated atmosphere surrounding the plant may be replaced by gases through the openings,  $H_2$  and  $O_2$ . During the course of an experiment the temperature did not vary more than  $1^\circ$  C.

Illumination during the determination of the normal orientation and the effect of mechanical stimulation was supplied by means of a 240-candle power frosted bulb, 1 meter above the aquarium. The light was allowed to fall directly on the plant through the glass window, W, in the bakelite cover of the aquarium. In the experiments on light two kinds of illumination were used: (1) illumination of the whole plant with equal intensities from all sides; and (2) illumination of a specific limited region of the plant axis. In obtaining illumination of the whole plant a Mazda type incandescent lamp of 240-candle power, placed at the distance of one meter above the aquarium, was used. The rays were reflected horizontally on the plant by four mirrors, one of which was placed on each side of the aquarium, tilted at  $45^\circ$  angles. Spot illumination was furnished by a galvanometer lamp and slit which focused the light to a band 1.5 mm. in width. A water filter 1.5 cm. thick between the plant and the lamp served to remove heat rays. The intensity of the light was regulated by an iris diaphragm in front of the lamp lens.

## Results

### NORMAL ELECTRIC POLARITY OF THE CENTRAL AXIS

In order to establish experimental reference conditions, an investigation of the normal inherent polarity was first made. The plant was transferred from the aquarium, in which it had been grown, to the plant holder. All operations were carried out under water with minimum mechanical stimulation. At no time did the plant come into contact with the dry atmosphere. The electrical potential between the apex, A, and the points noted by the letters on figure 1 B was obtained by lowering the water level of the aquarium by means of the rack and pinion, R. With such precautions in manipulation, the apex in the standard preparation was found to be electro-positive to the more basal regions. The regions of the greatest change in E. M. F. per unit length are in the first 5 mm. of the plant axis below the apex and across the first node, from C to D (fig. 1 B). In the second internode the electric polarity may at times be completely inverted, the apex negative to the base. In the third internode there is little difference in potential. In determinations made over a period of nine months the apex was positive to the more basal segments in 71 out of 78 plants tested. That is, in 91 per cent. of the cases tested, there was a positive potential gradient from apex to base.

The electric polarity of each plant was determined at intervals of two hours or more for 48 hours or longer and it was found that in the unstimulated condition the apex remained positive. There were a few exceptions to this rule as the apex became negative without obvious external stimulation during the first 24 hours. This was a temporary condition since in 6 to 12 hours the apex again became positive and remained so to the end of the experiment.

In *Chara* the maintained electrical polarity is in agreement with that of other organisms in that the apex, the region of greatest cell activity, is electropositive in the external circuit to the more basal regions.

#### EFFECT OF MECHANICAL STIMULATION ON THE ELECTRIC POLARITY

The purpose of the following experiments was to determine the effect of mechanical stimulation on the electrical polarity of the central axis. Two types of stimulation were impressed, first, continuous stimulation produced by a solid object, the glass contact, constantly touching the apex of the plant over an extended period of time; and, second, intense stimulation produced by the tap of an electromagnetic stimulator, which resulted in temporary mechanical deformation similar to that used by MARSH (20).

A slow inversion of the electrical polarity of the plant occurred when contact between the glass claw of the electrode and the apex of the plant was constantly maintained under water for an extended period of time. The electrical polarity was determined at intervals of two hours by the method previously described. It was found that this slight supporting pressure on the apex of the plant caused a complete inversion of the potential in about four hours. The electrical polarity returned to normal with the apex electropositive in about the same length of time when the glass claw contact was removed. Application of the contact a second time again caused the polarity of the plant to invert.

This experiment was repeated on 12 different plants, involving 29 cases of stimulation, and in all cases the polarity was decreased or inverted. The susceptibility of the electric polarity to slight mechanical stimulation demonstrated the delicate nature of the polarity potential and explains in part the inconsistent results obtained before perfecting the manipulative technique.

Diminution or inversion of the electric polarity by slight mechanical pressure might suggest an explanation of the various thigmotropic responses since much evidence has been obtained that change in morphological polarity of an organism is associated with change in electric polarity.

The device used for the determination of the effect of slight mechanical deformation applied for a short duration of time was a modification of the apparatus previously mentioned (20). It consisted of a signal magnet to

which was cemented a bent glass rod with one side ground to form a smooth flat surface. To the flexible spring another small bent rod was cemented forming, in effect, a small electromagnetic glass hammer which could deliver a sharp tap to any previously selected location on the plant axis. The stimulator was inserted in the aquarium through an opening covered by a rubber diaphragm. All adjustments were made by a micromanipulator. A roughly quantitative control of the strength of the stimulus was obtained by approximately varying the current which passed through the coils of the electromagnet. The time of application of the stimulus was one second. If any of the plants failed to grow after testing, or if microscopic examination revealed injury, the data from such experiments were discarded.

A mechanical stimulus at any point caused the stimulated region to become negative to the unstimulated region. The negative phase produced by this type of stimulation was of short duration, between 1 and 2 minutes. In a typical case the first two stimuli, applied at the apex 4 minutes apart, each caused a decrease of 15 millivolts in the potential between AD. The third stimulus caused a decrease of 14 millivolts and the fourth 13 millivolts. As a result of 12 successive stimuli of the same intensity, applied at intervals of 3 minutes, a negative response was obtained after each stimulus, and the potential change decreased from 15 to 3 millivolts, indicating "adaptation."

With the upper contacts at the apex and the lower at the base of the internode, stimulation at the apex decreases the polarity of the segment, and stimulation at the midpoint between the apex and base produces little or no change. Repetition of stimuli at corresponding points along the second node gave results similar to those of the first node but relatively smaller in magnitude.

In a typical case with the apical electrode at the midpoint (B, fig. 1, B) and the basal electrode at the base of the first internode (C, fig. 1, B), a decrease of 11.5 mv. in the polarity of the internodes was obtained when the stimulus was applied at the apex (A, fig. 1). There was no vibration of the plant in the water contact sufficient to produce this change. When the apical contact was replaced at the apex, and the plant stimulated at the apex, a decrease of 25 mv. was obtained. Essentially the same results were obtained on the second internode. This demonstrates that mechanical stimulation at the apex caused a temporary negative condition in the polarity of a segment 7 mm. from the point of application; therefore a wave of negativity was produced which traversed the distance of 7 mm. No evidence was obtained to indicate that the negative variation traversed the node. Because of the inadequacy of the electrometer to register rapid changes in potential, the phenomena of conduction will not be considered in the present paper.

## EFFECT OF LIGHT ON ELECTRIC POLARITY

When the living plant is illuminated, there is a change in the rate of output of electric energy by the plant. This fact was first observed by HAAKE (7) who found that the illuminated region became electropositive to the unilluminated portion and that no change occurred in etiolated petals and leaves under illumination. A. D. WALLER (29) suggested that a relation existed between photosynthesis and the electrical changes due to illumination. BOSE (4), using two halves of a banana leaf connected by a salt bridge, obtained changes in electrical potential when one of the leaves was illuminated.

J. C. WALLER (30-31) observed current changes between electrodes placed at illuminated and unilluminated symmetrical positions on the surface of leaves. According to him, the magnitude of the electrical response upon illumination is dependent on the species and age of the plant used. He stated that the "photoelectric" response in green leaves is dependent upon the photosynthetic process; however, he stated that, even in the absence of chlorophyll, current changes may be obtained upon illumination. Furthermore he attributed the changes in "current" incident to illumination to a lack of equilibrium between oxidation and reduction of a hypothetical "acid" in photosynthesis. He assumed that the positive phase of the current was due to the "reduction of the acid and that the negative phase was due to the oxidation of the acid." Unfortunately he used, as a measuring instrument, a galvanometer which does not allow a clear distinction to be made between a change in E. M. F. and a change in the resistance of the tissue between the electrodes.

SHEARD (27), working with the sunflower and the *Poinsettia* obtained potential changes as high as 0.3 volt on exposure of the leaves to the ultra-violet and infra-red light. He obtained no effect with illumination by light in the visible spectrum. GLASS (6) found that local illumination of the apex of the *Elodea* leaves by white light produced an increase in the potential of the apex with respect to the non-illuminated base.

The object of the following experiments is (1) to determine the effect of illumination on the electric polarity of *Chara*; (2) to establish the relation between the response upon illumination and the photosynthetic process; and (3) to relate this response in part, if not totally, to the increase in the oxygen available for intracellular oxidation.

In order to demonstrate that an electrical response produced by illumination is not due to spontaneous inherent changes in the polarity of the preparation, the first step was to determine the magnitude of inherent change, if any, in the polarity of the standard preparation in darkness, over an extended period of time under constant external conditions. The next procedure was to subject the whole plant to light, other conditions remaining the

same, and to observe the change in polarity produced. Since the final object was to determine the effect of continuous and discontinuous illumination of the apex, the interpretation of the results was simplified by observing the effects produced by illumination of one locus of the plant at any given instant.

In order to attempt to establish a relation between photosynthesis and the electrical response to light, the factors known to affect the photosynthetic process were varied and the results of these variations on the electrical potentials were observed. The variable factors were: (1) the intensity of illumination, (2) the wave length, (3) the concentration of carbon dioxide, and (4) the concentration of  $O_2$  in the atmosphere surrounding the plant. Discussion of the well known effects of variations of these factors on the photosynthetic activity is given by SPOEHR (28).

If the change of potential in the plant upon illumination is due to an increase of oxygen available for cell oxidation, the electrical response in light or in darkness should be characteristically modified by a change in oxygen tension around the plant. Therefore, to determine comparatively the effect of illumination on the plant in oxygen and in hydrogen (the absence of oxygen) should also be significant.

The plants prior to the experiment were allowed to remain submerged for two hours in the experimental aquarium in darkness so that any effect due to previous mechanical stimulation or a change of light intensity might have diminished or disappeared. Neither the time of the day nor the condition of illumination prior to experimentation could be shown to have any effect on the response of the plant to light. The periods when the plants were illuminated and unilluminated are indicated by the contrasting heavy lines at the bottom of the graphs.

#### PROCEDURE I. THE ELECTRICAL RESPONSE OF THE PLANT TO LIGHT.

1. *Normal fluctuation of the electrical potential of the standard preparation in air and darkness.*—The basal line of reference, which was assumed to be the normal E. M. F. residing in the unstimulated preparation in darkness and in vapor-saturated atmosphere, was determined. Figure 2 A shows the range of variability of two different typical plants under the above specified conditions during a period of 50 minutes. In neither of these preparations did any large variations of potential take place spontaneously. Records of 10 different plants over periods from 30 minutes to 2 hours show no greater change than 2 mv. per minute or a total of 10 mv. in 10 minutes. In most preparations a slow relatively small steady decrease in polarity occurred as shown by the broken curve in figure 2 A. Because of the previous results, which show that the presence of the contact at the apex of a plant causes an inversion of polarity in several hours, a decrease in the potential would



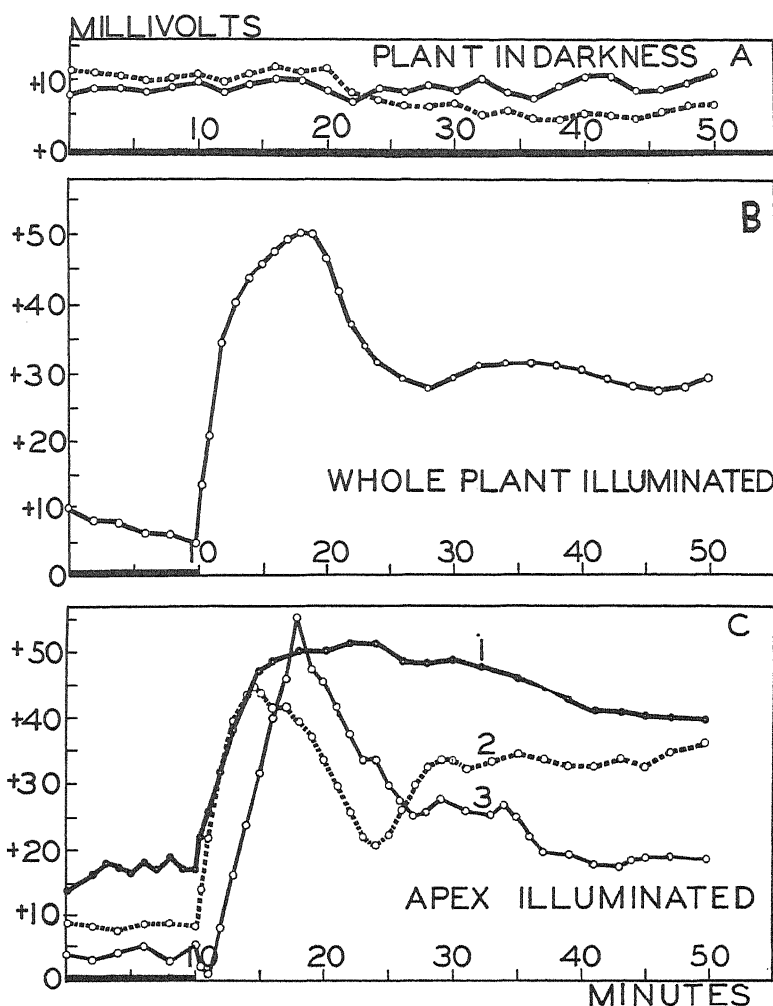


FIG. 2. A. The curves show the magnitude of the spontaneous variation of the E. M. F. of two typical standard preparations in darkness.

B. Effect of continuous illumination of the whole plant on the E. M. F. of the first internode. The light from a 240 c.p. incandescent lamp one meter above the aquarium was reflected on the plant by four mirrors placed on the four sides of the aquarium. The period of darkness before illumination is indicated by the heavy line at the base of the drawing.

C. Effect of continuous illumination of the apex only with light of 36,500 meter-candles on the E. M. F. of the first internode. Curves 1, 2, and 3 represent the responses of three plants under the same conditions.

be expected. *These results may be considered to represent the condition of the maintainance of flux equilibrium concentrations of the electromotively active constituents at a relatively constant value.* Either these curves or the readings taken in all experiments just before application of the light may serve as a control. In no cases were the inherent variations of E. M. F. of the same magnitude as those produced by illumination.

2. *Change in polarity caused by illumination of the whole plant.*—A typical example of the effect of illumination of the entire plant by light of the same intensity is shown in figure 2 B. For this purpose light from a 240-candle power Mazda bulb reflected by a mirror on the four sides of the plant was used. The curve shows a sharp rise in potential to a maximum of 51 mv. The potential sharply decreases to a lower relatively constant value. When the experiment was repeated on several different plants the same type of curves was obtained. In all cases the subsequently maintained polarity was greater than in darkness by at least 50 per cent. *The increase in potential upon uniform illumination affects unequally the potential of the apical and basal regions of the first internode.* This result has a fundamental significance and should be compared to the corresponding effects of oxygen, temperature, and anaesthetics on the electrical polarity in other forms.

3. *Increase of potential upon illumination of the apex only.*—In order to localize the region of greatest change in potential a series of experiments was carried out in which the apex only was illuminated with a spot of light 2 mm. in diameter immediately above the contact. In order to establish a reference level of polarity the potential was observed in darkness for 30 minutes before illumination. Upon illumination, there was an abrupt increase in potential followed by an irregular decline to a relatively constant level. The variability of this response is shown by curves 1, 2, and 3 of figure 2 B. Curve 1, which is typical of most cases, demonstrates an abrupt increase in potential upon illumination, followed by a slow decrease until a relatively high potential level is reached and maintained. In the plant in curve 2, the potential decreases rapidly after the initial difference. Curve 3, characteristic of some cases, illustrates an abrupt increase to a maximum followed by an irregular decrease to a lower constantly maintained polarity. The small initial decrease followed by a steep rise upon illumination, as shown in curve 3, has been obtained in many experiments.

With the claw contact at the apex and the water meniscus just below the first node and with the plant in darkness constant polarity potential of plus 10.0 mv. was obtained. The plant was returned to water and the apex illuminated 10 minutes under water. At the end of this time, with the light still on, the plant was again tested and the potential of the same segment had increased to plus 42 mv. Therefore the increase in potential on illumination is not a phenomenon which takes place only when the plant is out of water and in air.

4. *Electrical polarity of the plant during alternate exposures of the apex to light and darkness.*—Figure 3 A shows the changes which occur in the

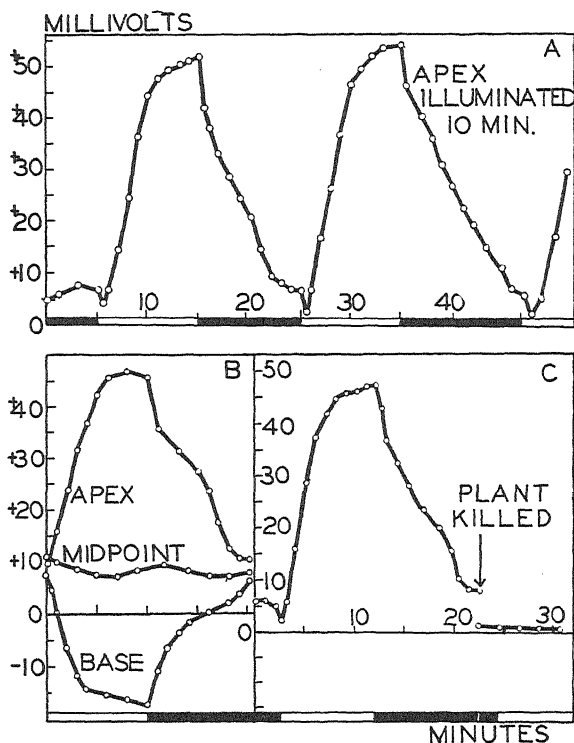


FIG. 3. A. The effect of alternate exposure of the apex to light of 36,500 meter-candles and darkness on the E. M. F. of the first internode. The periods when the light was applied are indicated by the white lines at the base of the drawing.

B. The change in electric polarity of the first internode when different loci along the central axis were illuminated. The curves obtained upon illumination of the different loci are labelled.

C. Absence of the effect of illumination on a dead preparation. The first response was obtained before the plant was killed.

plant's potential during alternate 10-minute exposures of the apex to light and darkness. The illumination for each 10-minutes period was  $21.9 \times 10^7$  meter-candle-seconds. The periods of darkness are indicated by black bars at the base of the figure, while the illuminated periods are represented by light bars. Illumination produces an immediate decrease, which is followed by an increase of 50 mv. in the potential of the apex with respect to the base. When the light is shut off, there is a drop in potential, and the polarity returns to the original level in darkness. The effect could be repeated a number of times with a duplication in the type of curve, and little variation

in the magnitude of the responses. Within these time limits no evidence of "fatigue" as reported by A. D. WALLER (29) was obtained.

5. *Effect of illumination of different loci along the plant axis.*—Illumination of a given point along the plant axis renders it more positive. When contacts are located at the apex and just below the first node, illumination of the apex causes an increase in the inherent polarity. If the base is illuminated, it becomes more positive. Hence, there follows an inversion of the polarity of the system, which causes the apex to become relatively negative to the base. When the midpoint between apex and base is illuminated there is either no change or a minimum change in the polarity potential. That a change actually occurs in the midregion may be shown by moving the contact to the midpoint and illuminating that area, thereby producing an increase in the local potential at this midpoint. One may further show that a change has occurred by measuring the potential between the apex and midpoint, and returning the contact and measuring the potential. Both methods on the same plant showed an increase of 8 mv. in the illuminated region. This is comparable to the case, discussed earlier in this paper, in which mechanical stimulation produced a decrease of potential at the midpoint but caused no change in the total potential of the internode.

With the claw contact at the midpoint of the first internode, and the water meniscus just below the first node, no change in the potential was obtained when the apex was illuminated for 10 minutes. When the claw contact was replaced at the apex of the same plant, and the apex illuminated for 10 minutes, an increase of 42 mv. was obtained. These results indicate that under the conditions of this experiment illumination outside the area of the contacts produces no change in the potential between the contacts.

6. *Effect of killing the preparation on its response to light.*—Death of the plant abolishes all responses to illumination. Figure 3 C shows the normal response of a first internode of a living plant to apical illumination. At the point designated on the graph, the plant was removed from the instrument and immersed for an instant in boiling water. When the plant was replaced in the instrument, with the electrodes at the same position, no change in potential occurred upon apical illumination. Plants killed by chloroform and formaldehyde exhibited no polarity and gave no response to light.

## PROCEDURE II. CHANGE IN POLARITY WITH VARIATION OF EXTERNAL FACTORS KNOWN TO AFFECT PHOTOSYNTHESIS

1. *Effect of the intensity of illumination on the electrical polarity of the standard preparation.*—A. Discontinuous illumination of the whole plant with light of varying intensity.—Light from globes of different candle power was reflected through water filters by means of the four symmetrical mirrors

arranged as previously described. The claw contact was at the apex, and the water meniscus contact just below the base of the first node of the standard preparation. Illumination was supplied by different lamps for the same length of time. The periods of illumination are represented by the light bars, and the periods of darkness by the dark bars at the base of figure 4 A.

The plant was illuminated at the points A, B, C, and D in the curve from bulbs of 18-, 90-, 185-, and 240-candle power, respectively, placed one meter above the aquarium. With the lowest intensity of illumination, 18-candle power, at A, little response was obtained. When the lights with increased candle power were used there was an increase in the magnitude of the response. Attention is called to the inversion of polarity which takes place when the light is turned off, which result will be explained later. After each increased response, there is an increase in the magnitude of the inversion. When the plant was allowed to remain in darkness at the end of the experiment, the potential returned to the normal base level.

B. Discontinuous illumination of the apex with light of varying intensity.—Figure 4 B shows the effect of increasing the intensity of apical illumination on the polarity of the standard preparation. The light was applied for 10-minute intervals followed by 10-minute periods in darkness. No change was obtained at A when light of the intensity of 486 meter-candles was used. A change of 20 mv. took place upon illumination with light of 1215 meter-candles. At the points on the graph indicated by D, E, and F, apical illumination of 11,500, 17,100, and 36,500 meter-candles produced responses of 50, 52, and 54 mv. respectively.

Examination of the data shows that there is a lower threshold of light intensity below which no change is produced upon illumination. Above this threshold a relatively small change in luminous intensity produces a large increase in polarity. An increase of the illumination above 17,000 meter-candles produced but little increase in the response of the plant.

C. Continuous illumination of the apex with light of varying intensity.—A potential increase of 5 to 52 mv. produced by illumination with 36,500 meter-candles was obtained between the base and the apex of the first internode of a standard preparation (fig. 4 C). At 10-minute intervals the light intensity was decreased by decreasing the diameter of the effective aperture of the lens. The decrease of illumination at B to 17,100 meter-candles produced no significant decrease in the potential. Reduction of the light-intensity at C to 11,500 meter-candles caused a decrease of 7 millivolts in the polarity. The reduction of the polarity at D, E, and F of 11.5, 15, and 10 mv., respectively, was due to a decrease of the light intensity of 3650, 1215, and 486 meter-candles, respectively. The decrease at G from 486 meter-candles to complete darkness produced no apparent effect. When light was increased, as shown by the ascending part of the graph, there was

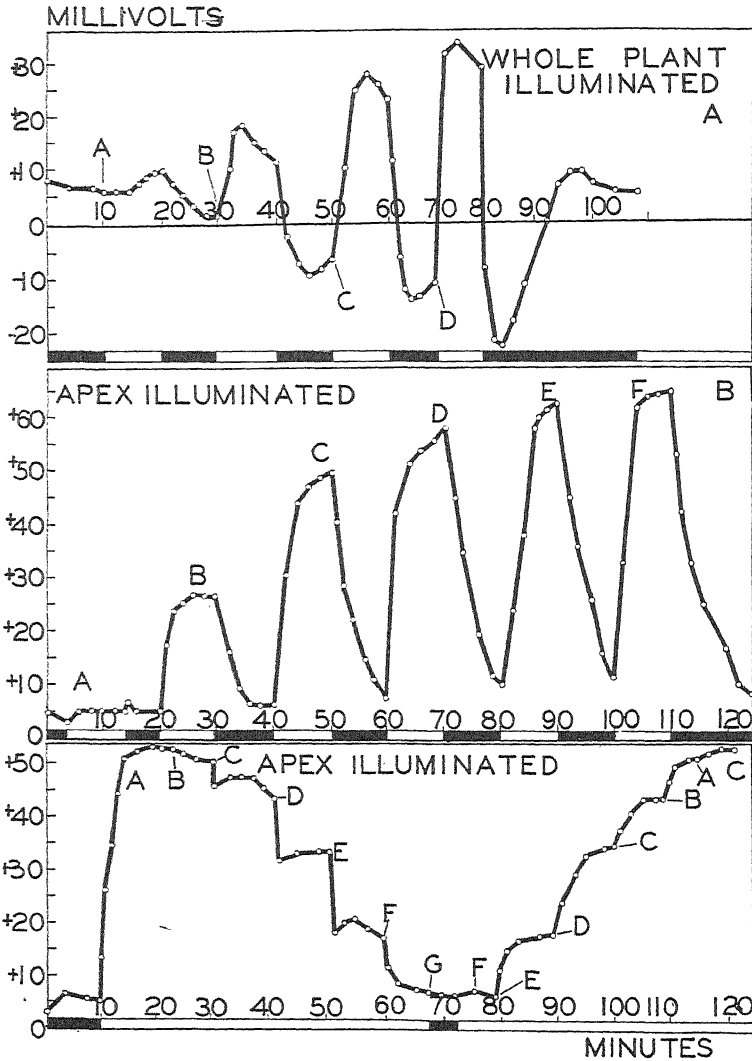


FIG. 4. A. The effect of discontinuous illumination of the whole plant with increasing intensity of light. The light was reflected on the preparation by mirrors on the four sides of the aquarium. Illumination at A was with an 18-c.p. globe at 1 meter, B with 90, C with 185 and D with 240-c.p. globes, respectively.

B. The effect of discontinuous apical illumination of increasing intensity. The apex was illuminated at A with 486 meter-candles, at B with 1215, at C with 3650, at D with 11,500, at E with 17,100, and at F with 36,500 meter-candles, respectively. The periods of illumination are indicated by the unshaded regions of the line at the base of the figure.

C. The control of the magnitude of polarity by decreasing and increasing illumination of the apex. The apex was illuminated with 36,500 meter-candles. At B the intensity of illumination was decreased to 17,100, at C to 11,500, at D to 3650, at E to 1215, at F to 486 meter-candles and at G to complete darkness. In the second half of the ... the illumination ...

a corresponding increase in the potential difference of the base to the apex. From these data one may assume that, with other conditions the same, the level of electrical polarity in the plant is largely a function of the intensity of light on the more apical region. By controlling illumination the electric polarity may be decreased to any desired level.

2. *Discontinuous light of different wave lengths.*—Light of different wave lengths was obtained by placing Corning colored glass filters between the plant and the source of illumination, as designated by C in figure 5. The

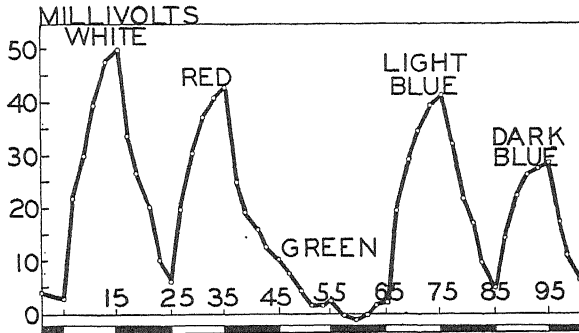


FIG. 5. The effect of stimulation of the apex with light of different wave lengths on the E. M. F. of the first internode. The color of the filter used is stated above each response. The wave length of the light transmitted by the filter marked "red" is 649 to 740  $m\mu$ , "green" from 490 to 555  $m\mu$ , "light blue" from 445 to 505  $m\mu$ , "dark blue" from 415 to 490  $m\mu$ . The time of application of light can be ascertained from the unshaded lines at the bottom of the figure.

wave length of the light transmitted by the dark blue filter was from 415  $m\mu$  to 490  $m\mu$ , by the light blue filter from 445 to 505  $m\mu$ , the green filter from 490  $m\mu$  to 550  $m\mu$ , and the red filter from 649 to 740  $m\mu$ .

Light from a tungsten filament lamp filtered through clear glass and falling on the apex of the standard preparation produced a positive increase of 47 mv. labeled "white" in figure 5. Red light of the wave length 649 to 740  $m\mu$  produced a change of the same type but only 38 mv. in magnitude. Light passing through the light blue filter (wave length 445 to 505  $m\mu$ ) produced an increase of 40 mv. and the dark blue (414 to 490  $m\mu$ ) caused an increase of 22 mv. Green light (490 to 555  $m\mu$ ) failed to produce any change. Repetition of the above experiment failed in all cases to give an increase in the E. M. F. with green light. These experiments indicate only qualitatively the effect of light of different wave lengths, since neither the intensities nor the light energies present in the light of different wave lengths are comparable.

3. *Effect of  $CO_2$  on the polarity with apical illumination.*—With the light intensity used in these experiments, the concentration of  $CO_2$  was the limiting factor (29). An increase in  $CO_2$  in constant illumination should speed

up the rate of photosynthesis, subsequently producing an increase in the potential of the illuminated region, if the rise in intracellular concentration of  $O_2$  is responsible for the increase in magnitude of potential. The effect of  $CO_2$  on the polarity of a standard preparation in darkness in air was first determined. When 1 per cent.  $CO_2$  by volume was introduced into the space above the water level in the aquarium, no effect was produced in the polarity of the plant as shown in figure 6 A. Upon illumination of the apex of a plant in water-vapor saturated air, the potential increased to a maximum;

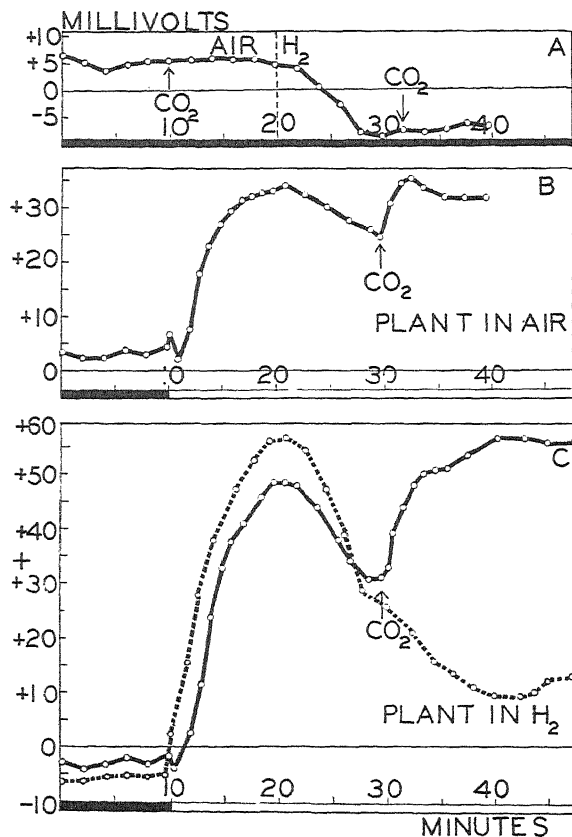


FIG. 6. A. The effect of carbon dioxide on the E. M. F. of the first internode in air and in hydrogen in darkness. The time of application of 1 per cent. carbon dioxide is indicated by the arrows. The dotted line shows the time the gas surrounding the plant was changed from air to hydrogen.

B. The rise of potential on increasing the carbon dioxide concentration in the air surrounding the plant, the apex of which was constantly illuminated. The time of the introduction of 1 per cent. carbon dioxide is indicated by the arrow.

C. The broken curve shows the effect of continuous illumination of the apex in an atmosphere of hydrogen. The solid line represents a comparable preparation under the same condition except that 1 per cent. carbon dioxide is added at the time indicated.



then it fell to a constant level as in the other cases previously discussed. Now, with the introduction of  $\text{CO}_2$  gas in light (fig. 6 C), there was in this case an abrupt increase in potential. After the initial overshooting, the potential dropped slightly but was maintained at a new level higher than the original.

The broken curve in figure 6 C shows the electrical behavior of the first internode of a standard preparation in hydrogen, with apical illumination. The plant was allowed to remain 30 minutes in an atmosphere of hydrogen before the first reading was recorded. The initial polarity as shown by the graph was inverted. The initial potential rapidly increased upon illumination to a maximum of 55 mv. This increase was followed by a decrease to a lower level than usually is attained by preparations under the same condition in air. The continuous curve represents a comparable preparation under the same experimental conditions, except that 1 per cent.  $\text{CO}_2$  was introduced at the point indicated on the curve. The effect produced was an immediate increase of the potential to a constant high level. Since the rate of photosynthesis depends on the concentration of  $\text{CO}_2$ , the process is speeded up when the concentration of  $\text{CO}_2$  is increased. This increase in photosynthesis evidently raises the concentration of oxygen and other electromotively active substances available for cell oxidation and therefore for the maintenance of electrical polarity.

### PROCEDURE III. EFFECT OF OXYGEN TENSION ON THE ELECTRICAL RESPONSE TO ILLUMINATION

1. *Effect of oxygen tension on the standard preparation in darkness.*—In this experiment, the claw electrode contact was at the apex and the water meniscus contact at the base of the first internode of the standard preparation. The gases, which were saturated with water vapor by passing them through Bunsen columns filled with distilled water, were passed at a constant rate through the space above the water level in the aquarium. Examination of figure 7 A shows that in hydrogen in darkness a decrease of approximately 20 mv. was obtained with an inversion of polarity. Incidentally, it is impossible to obtain an atmosphere about the plants entirely free of oxygen since dissolved oxygen from the water was continuously passing into the atmosphere above the water. However, in spite of this fact the results are perfectly clear. When oxygen was introduced, there was a subsequent increase of 30 mv. in the polarity potential, followed by a decrease and a subsequent rise to a constantly maintained level of potential. Repetition on several different plants gave essentially the same results. These curves are strikingly similar to those obtained on the effects of presence and absence of oxygen on the potential in the onion root and may be interpreted in the same manner (ROSENE and LUND, 25).

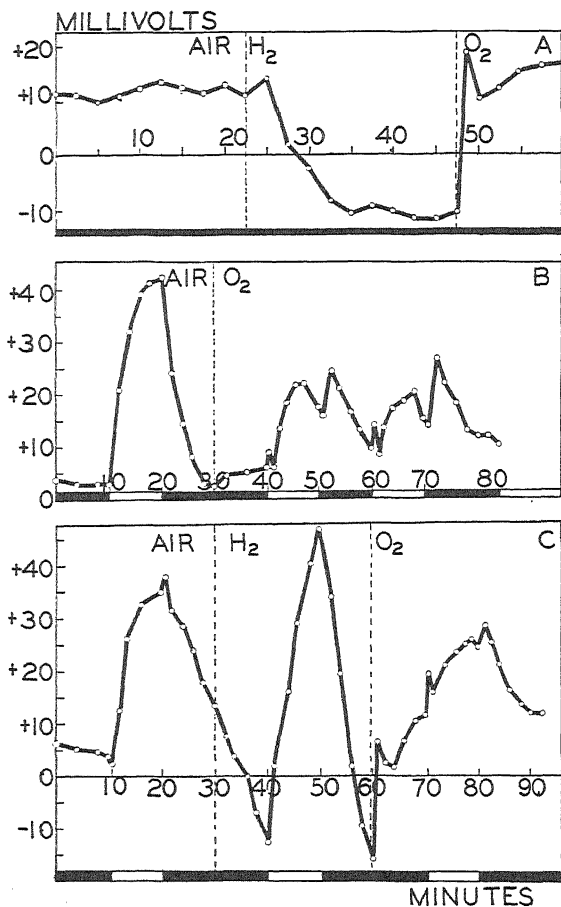


FIG. 7. A. The effect of (a) air, (b) hydrogen, and (c) oxygen on the polarity of the standard preparation in darkness. The times of change of the gases are shown by the dotted lines.

B. The response produced by illuminating the apex of the standard preparation in atmospheres of air and oxygen. The time of application of the light may be determined from the unshaded portions of the line at the base of the plate. Oxygen was introduced at the time indicated by the dotted line.

C. The change in E. M. F. of the standard preparation produced by apical illumination of the standard preparation in atmospheres of air, hydrogen, and oxygen. The period of illumination is indicated by the unshaded portions of the line at the base of the drawing. The broken line shows the time of change of gases.

2. *Comparison of the modifications of the response due to illumination in an atmosphere of oxygen, hydrogen, and air.*—Figure 7 B shows the effect of illumination on the standard preparation, first in air and then in oxygen, the intensity and time of the illumination being the same in both cases. A

typical response of 42 mv. was obtained with the plant in air. When the atmosphere about the plant, in darkness, is changed from air to oxygen, there is a small increase in potential. Illumination in oxygen causes an initial rise and fall of only 2 mv., followed by a potential increase. The potential reaches a maximum and then decreases. Upon discontinuing the illumination there is an immediate rise in the potential followed by a decrease to a lower level than in light. In air, there is an increase in potential of 41 mv. compared to 15 and to 9 in oxygen.

Figure 7 C shows the effect of light on the apex of the standard preparation in an atmosphere of air, pure hydrogen, and pure oxygen. In air, the illumination produced an increase of 35 mv. in the polarity of the first internode. In hydrogen the same illumination produced an increase of 59 mv., while in oxygen only a 15 mv. response was obtained. *Attention is called to the decrease in potential when hydrogen replaced the air in darkness, and subsequent increase in potential when oxygen replaces the hydrogen in darkness.*

### Discussion

The purpose of the first part of this investigation was to establish a relation between light and the maintained polarity in *Chara*. The change upon illumination cannot be attributed to an increase in temperature, as the heat rays are filtered out by the water. The ultra violet rays are removed by the glass. Therefore any change must be due to the effect of light of the visible spectrum. Visible light could affect either the photosynthetic process or some unknown photoreceptive mechanism in the plant.

An increase in the intensity of illumination increases the electric polarity.<sup>1</sup> An increase in CO<sub>2</sub> concentration in light increases the electric potential. Green light produces no change in the E. M. F. of the system, while red and blue light both caused an increase in the potential. Likewise, in green plants (29) the increase in intensity of illumination and the concentration of CO<sub>2</sub> increases the rate of photosynthesis; also, in green light photosynthesis is at a minimum. Since all these facts are consistent with one another, one may logically conclude that photosynthesis is closely linked with the increase in the electrical potential due to illumination. Furthermore, from the following facts, it becomes practically certain that the effects of light on the E.

<sup>1</sup> The electrical polarity of a single cell is

$$E_p = -RT/2F \ln P_{c_1}/P_c = -RT/2F \ln r',$$

where R is the gas content, T, the absolute temperature, ln, the natural logarithm, F, the faraday equivalent, and P<sub>c</sub> the oxygen pressure at a given locus inside the cell, and r' is the ratio of P<sub>c<sub>1</sub></sub> to P<sub>c</sub>. The electrical polarity of a polar tissue may be expressed as:

$$E = \Sigma E = -RT/2F \ln r' \text{ apical}/r' \text{ basal.}$$

P<sub>c</sub> has been shown to be quantitatively related to P, the pressure of oxygen surrounding the tissue.

M. F.'s are primarily caused by changes produced in the cell by photosynthesis.

1. Oxygen is released by photosynthesis.
2. A quantitative relation exists between the output of electric energy and cell oxidation in all polar tissues which have been investigated up to the present.
3. The quantitative relations between oxygen tension, velocity of  $O_2$  consumption, and electric potential in the onion root have been formulated by LUND (17) on the basis of flux equilibrium in cell oxidation.

Using LUND's fundamental assumptions (12) as a basis for his work MARSH has recently extended this formulation, along with direct experimental evidence, to show how oxygen concentration is related to the inherent E. M. F. In such a system the loci may be considered to act essentially as modified oxygen electrodes. The above conclusions of course do not exclude the possibility that other products of photosynthesis, besides free oxygen, also affect the potential.

The rate of production of oxidizable substance, which is equivalent to the oxygen consumption at flux equilibrium, has been shown to be greater in the apical than in the basal regions of various tissues (12, 17, 21). In view of this fact, we should expect the increase in potential at the locus of the apex, upon illumination of the apex, to be greater than that in the base. This is precisely what was observed in the experiments on the illumination of the whole plant (fig. 2 B), and with illumination of the apex and base separately (fig. 3 B).

The inversion of polarity (fig. 4 A) after illumination of the whole preparation can be explained by the difference in the rate of oxygen consumption of the apex and the base. If  $O_2$  were produced by photosynthesis at approximately equal rates at both apex and base, an unequal change in the potential at the two loci would result, since it has been found that the same change in concentration of oxygen at apex and base causes a greater change in the apical than in the basal tissues (26). The increased consumption of oxygen, continuing at the apex after the light is removed, would cause a relative decrease in the electrical potential. Since the oxygen is consumed at a lesser rate in the base, it would be available for a longer period of time with the consequent maintenance of a relatively higher potential. The higher positive potential at the base would result in the observed inversion of the polarity of the internode (fig. 4 B). As the oxygen is consumed in the cell oxidations, the polarity gradually reverts, reaching the constant potential level in darkness.

This conception adequately explains the increase in the electrical response in the absence of oxygen. A decrease in oxygen tension around the polar

tissue leads to an inversion of polarity which is in accord with the present observation (fig. 13 A) and with the results of ROSENE and LUND (25) and with MARSH's mathematical formulation (19) of the relation between the flux equilibrium oxygen pressure,  $P_c$ , and the oxygen pressure,  $P$ , of the atmosphere surrounding the plant. In low concentrations of oxygen as all the available oxygen is used up by respiration of the plant, the potential is decreased at both the apical and basal loci, the potential of the apex being decreased relatively more than at the base. Upon apical illumination the potential of the apex is again increased relatively more than that of the base, thus accounting for the increase in the apical-basal potential. The potential of the apex apparently decreases (as illustrated in the dotted line of figure 6 C) because of the decrease in the photosynthetic rate due to the removal of  $\text{CO}_2$ .

Even with the plant in oxygen,  $P_c$  does not necessarily equal  $P$ . Moreover, upon illumination  $P_c$  might be further increased. With the plant in oxygen, the absolute value of the apical  $P_c$  and the basal  $P_c$  are greater; hence, an increase in the apical  $P_c$  up to its maximum does not change the ratio of the apical  $P_c$ /basal  $P_c$  as much as when the basal flux equilibrium oxygen pressure has a lower value. This is demonstrated in figure 7 B. The method used here may serve to distinguish  $P_c$  from  $P$ .

Even though the general results obtained on *Chara* may be explained as the effect of an increased production of oxygen in the polar tissues, it is probable that the observed changes in the electrical potential may also be partly determined by other concomitant electrochemical processes in the cell. CLARK (5) and WILKS (*loc. cit.*) report definite electrical changes due to illumination in the etiolated oat coleoptile which, in the absence of chlorophyll, are not due to photosynthesis. WALLER reports "photoelectric" changes in the etiolated petals of some plants. It is also highly probable that electromotively active substances other than oxygen are produced in the photosynthetic process.

The experimental results have shown that light when appropriately applied may act differentially and quantitatively to increase and decrease reversibly the electric polarity and the potential at any locus on the main axis in *Chara*. In this respect the effect of light is similar to the effect of temperature, and of the concentration of oxygen around the tissue, both of which are factors influencing cell oxidation. The output of electrical energy appears to depend on the flux equilibria between the substances necessary for cell oxidation and the concentration of oxygen. Both of these may act as limiting factors. By photosynthesis under certain conditions the chloroplasts supply the oxygen necessary for the intracellular oxidation and the maintenance of the electrical polarity of the cell.

### Summary

1. An apparatus is described for measuring the electrical polarity of *Chara vulgaris* under a minimum of mechanical disturbance.

2. The apex of *Chara vulgaris* is electropositive in the external circuit to the more basal regions. In the unstimulated condition the apex remains positive over a period as long as 48 hours.

3. The slight pressure exerted by a glass contact supporting a plant by the apex under water causes an inversion of the electric polarity. The apex returns slowly to the electropositive condition when the contact is removed.

4. Mechanical stimulation causes the E. M. F. of the stimulated region to become more negative. The change in E. M. F. produced is reversible, returning to the unstimulated condition in about two minutes. Stimuli applied outside the area between the electrode contacts may produce a change in the potential in the region of the plant included between the contacts. No evidence of transmission of the electrical effect of stimulation across the node was obtained.

5. Illumination of the whole plant increases the polarity of the apical region relatively more than the basal regions. The electropositivity of all illuminated regions is increased. The electric response to illumination does not occur in a dead plant.

6. The following facts relate the change of potential upon illumination to photosynthesis: (a) increase of the response with increased intensity of illumination; (b) failure to obtain an increase in potential on illumination with green light, while an increase was obtained in red and blue light; (c) an increase in polarity in light and absence of  $O_2$  with an increase in the carbon dioxide concentration.

7. In low oxygen tension, the response to light is increased; in high oxygen tension it is decreased.

8. The results can be explained on the basis of the equations formulated by MARSH (22) for the relation of the flux equilibrium oxygen tension and electric polarity.

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UNIVERSITY OF TEXAS  
AUSTIN, TEXAS

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# A STUDY OF MINERAL NUTRITION OF WHEAT AS INFLUENCED BY FERTILIZER COMBINATIONS<sup>1</sup>

FRANK T. DONALDSON

(WITH TEN FIGURES)

## Introduction

Practically the only method of following the movement of mineral nutrients into field crops consists of measuring the actual weights of the various elements present at progressive stages of growth. To do this it becomes necessary to determine not only the percentage composition of the plant, but also the average weight per plant or per tiller. This type of procedure was followed in the investigation described in this paper in an effort to determine the effect of various fertilizer combinations upon the rates of assimilation and the amounts of mineral nutrients entering the wheat plant.

There are comparatively few instances of the application of progressive development studies to wheat in which the whole plant has been studied. One of the earliest investigations of this type was published by WILFARTH, RÖMER, and WIMMER (15) in 1903. In this work samples were gathered from a field of spring wheat on four successive dates and analyses made for starch, potash, sodium, nitrogen, and phosphorus. The data were expressed as percentage of dry matter and pounds per acre.

In 1931 KNOWLES and WATKIN (9) published a thorough and comprehensive investigation of the nutrition of winter wheat. Nine successive samples were gathered over a period extending from seven weeks before ear emergence until harvest. The value of their data is enhanced by the large sample of 3200 tillers that was gathered at each sampling date. From the fourth to the ninth samplings the heads were separated from the leaves and stems and a further division was made of the eighth and ninth samplings into grain and chaff. Their results are expressed as the weights of substances present in 3200 tillers.

While the present investigation was in progress WOODFORD and MCCALLA (16) published the results of their investigation of the nutrition of spring wheat grown on the gray and black soils of Alberta. Their method of procedure was essentially the same as that of the English workers, although their results are expressed as the weights of nutrients present in plants taken from a unit length of row. Since the black soil contained four times the nitrogen and twice the phosphorus of the gray soil, their study, in part,

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demonstrates the effect of varying amounts of mineral nutrients upon the nutrition of the wheat plant and parallels more closely the work described in the present investigation.

### Materials and methods

#### CONDITIONS UNDER WHICH CROP WAS GROWN

A plot of ground was selected for this investigation in a locality known to possess rather infertile soil, in order that the response to the added fertilizers might be accentuated. This soil was a light, sandy loam, low in organic matter and total nitrogen and deficient in phosphorus. An analysis of the soil is given in table I.

TABLE I

CHEMICAL ANALYSES OF SOIL UPON WHICH WHEAT WAS GROWN

SUBSTANCE IN SOIL	FIRST FOOT	SECOND FOOT
Phosphorus (percentage) .....	0.061	0.063
Calcium oxide (percentage) .....	1.32	5.40
Total nitrogen (percentage) .....	0.059	0.042
Available phosphorus (Winogradsky test).....	deficient	.....
Nitrate nitrogen (p.p.m.) .....	3.00	6.00

The ground chosen for the plots was planted to potatoes the previous year. The potatoes were never irrigated and, since they received only one cultivation late in the summer, the weeds made a dense growth. While this condition made necessary considerable weed pulling the following year, it no doubt insured the removal of most of the nitrates from the soil, thus providing an opportunity to study the effect of adding nitrates.

A detailed account was kept of weather conditions at the site of the plots and these observations are augmented by the data from the cooperative weather observation station at Bozeman, 22 miles distant. Table II lists the pertinent weather and crop data through the growing season and the dates upon which sample collections were made.

Six one-twentieth-acre plots were seeded. Five of these were fertilized with the various fertilizer combinations shown in table III and one was left unfertilized for a check.

The potassium chloride was of the strength known as 50 per cent.  $K_2O$ ; the sodium nitrate contained 15 per cent. nitrogen; and the treble superphosphate 43 per cent. available  $P_2O_5$ .

#### SAMPLING

In all cases samples were collected (table II) before ten o'clock in the morning to avoid the effects of any rhythmic variation in nutrient assimilation which might occur during the day. Plants were pulled from the rows in the

TABLE II  
WEATHER AND CROP DATA

DATE OF COLLECTION	WEATHER AND CROP DATA THROUGHOUT GROWING SEASON
1936	
March 1–May 1....	The precipitation record at Bozeman shows 1.6 inches for this period compared with the 36-year average of 2.9 inches
May 2–May 13.....	A good rain fell in the vicinity of the plots on May 7. Bozeman Station recorded 0.85 inch On May 13 mineral fertilizers were applied to the plots and Marquis wheat seeded
May 14–June 25	Between May 15 and May 19 there were general rains which insured good germination of the seed On May 22 the wheat was just up and appeared in excellent condition Rain on June 1, 2, 3, 5, 6, and 7. Rains on June 5, 6, and 7 represented the last precipitation of any benefit during remainder of growing season On June 16 wheat in all plots stood 3 to 4 in. high and soil was moist to a depth of 18 in. On June 25 plots were sampled for first time. Wheat stood 6 to 10 in. high
June 26–July 6....	Hot, dry weather during this period evaporated soil moisture at a rapid rate Plots were sampled for second time on July 6. Although the ground was quite dry the plants appeared to be in excellent condition. None of wheat had started to head
July 7–July 16.....	An attempt was made to irrigate the plots but the demand for water on the farms with prior water rights was so great that there was not sufficient head to raise the water into the field ditch On July 16 wheat was sampled for the third time. Wheat on all plots had headed out and showed some damage from the dry weather
July 17–July 24	On July 21, when the demand for water had lessened somewhat, plots were irrigated. Three days later, July 24, the fourth set of samples was collected
July 25–July 31	Fifth set of samples was collected on July 31. The kernel was in the firm dough stage at this time
Aug. 1–Aug. 7....	On August 7 the sixth and last set of samples was gathered. Wheat was ready to harvest and was cut the following day

plots in one-foot sections in such a manner that the evacuated spaces formed a series of steplike patterns in the plot. No plants were used from the two outside rows nor from three-foot sections at the ends of the plots.

TABLE III  
FERTILIZERS APPLIED TO THE ONE-TWENTIETH-ACRE PLOTS

PLOT NO.	KINDS AND AMOUNTS OF FERTILIZERS
1 .....	None
2 .....	10 pounds KCl, 12 pounds $\text{NaNO}_3$ , 20 pounds treble superphosphate
3 .....	10 pounds KCl, 20 pounds treble superphosphate
4 .....	20 pounds treble superphosphate
5 .....	12 pounds $\text{NaNO}_3$
6 .....	10 pounds KCl, 12 pounds $\text{NaNO}_3$

Only an approximate count was made of the plants at the time of sampling, but an effort was made to have the sample contain from 200 to 300 plants. As soon as each collection was completed, the roots were wrapped in moist cheese cloth and the samples transported to the laboratory at Bozeman. Upon arrival at the laboratory the plants were counted and the roots cut from the plant one inch above the ground level and discarded. In the last three samples the heads were separated from the leaves and stems for separate analysis. The samples were then placed in a large oven held at 65° C. and equipped with a forced air draft. After drying for 24 hours the samples were removed to the open room and allowed to come to equilibrium with the moisture of the atmosphere. They were then weighed and ground in a Wiley mill to pass a 1-mm. screen.

#### ANALYSES

**DRY MATTER.**—Moisture determinations were made on the air-dried material. The samples were held 5 hours in a vacuum oven at 98° C. The percentages of all the nutrients determined are based on the dry matter as determined by this procedure.

**NITROGEN.**—Total nitrogen was determined by the Kjeldahl method with metallic mercury as a catalyst. A modification of the reduced iron method of PUCHER, LEAVENWORTH, and VICKERY (12) was used on the samples collected at the first two sampling dates to include nitrate nitrogen.

**PHOSPHORUS.**—Phosphorus was determined gravimetrically by precipitation as magnesium ammonium phosphate after first digesting the sample in a mixture of perchloric and nitric acids (5, 6).

**SULPHUR.**—An aliquot of the perchloric-nitric acid digest was neutralized with ammonia, made slightly acid with hydrochloric acid, and the sulphates precipitated with barium chloride.

**POTASSIUM.**—Potassium from a third aliquot of the acid digest was determined by the perchloric acid method (7, 8).

**ASH.**—The sample was burned over night in a platinum dish at a temperature of approximately 560° C.

**SOLUBLE ASH.**—Soluble ash was determined as that part of the ash soluble in N/2 hydrochloric acid.

#### PRESENTATION OF RESULTS

A unit of one plant was chosen as the basis for calculating the results of this study. The total dry weight of each sample was divided by the number of plants in the sample to obtain the average weight per plant. The product of this value and the percentage composition is expressed in milligrams per plant for all data. The term "whole plant" is used to designate the entire aerial portion of the plant. Since the heads were not counted, the data given for heads are based on the average weight of heads per plant.

TABLE IV

AVERAGE AMOUNTS OF SUBSTANCES IN ENTIRE AERIAL PORTION OF PLANT  
EXPRESSED AS MILLIGRAMS PER PLANT

Plot	FERTILIZER TREATMENT	SUBSTANCES IN PLANT ON DATE OF SAMPLING					
		JUNE 25	JULY 6	JULY 16	JULY 24	JULY 31	AUG. 7
		mg.	mg.	mg.	mg.	mg.	mg.
Dry matter							
1	Check	433	1080	1440	1620	1790	1910
2	NPK	584	1240	1730	1970	2110	2250
3	PK	521	1080	1510	1820	2030	2060
4	P	562	1270	1630	1920	2150	2280
5	N	507	1070	1480	1790	1970	2110
6	NK	431	1000	1520	1720	1990	1970
Phosphorus							
1	Check	1.18	2.54	2.64	2.82	3.14	3.69
2	NPK	1.65	2.86	3.06	3.50	3.50	3.91
3	PK	1.42	2.59	2.93	3.10	3.57	3.84
4	P	1.53	3.05	3.18	3.12	3.76	4.37
5	N	1.27	2.21	2.50	2.84	3.10	3.57
6	NK	1.12	2.11	2.40	2.91	3.22	3.22
Sulphur							
1	Check	1.25	2.93	3.11	3.35	3.42	3.70
2	NPK	1.52	2.96	3.18	3.53	3.57	3.78
3	PK	1.30	2.65	2.88	3.41	3.47	3.65
4	P	1.55	3.35	3.31	3.64	3.90	4.10
5	N	1.43	2.70	2.84	3.46	3.47	3.95
6	NK	1.15	2.42	2.78	3.28	3.65	3.85
Nitrogen							
1	Check	15.0	25.7	25.8	25.0	26.5	27.1
2	NPK	20.8	28.9	30.6	31.6	31.0	32.9
3	PK	17.5	24.9	26.6	28.2	30.1	29.3
4	P	19.1	29.5	28.9	30.6	31.6	32.6
5	N	18.9	27.3	27.0	28.8	29.4	30.9
6	NK	16.1	25.0	27.2	27.4	29.7	28.2
Calcium							
1	Check	1.99	3.63	3.50	3.58	3.87	3.86
2	NPK	2.59	4.10	3.93	4.63	4.43	4.62
3	PK	2.28	3.56	3.37	3.99	4.05	4.14
4	P	2.59	4.62	4.08	4.56	4.49	4.45
5	N	2.32	3.43	3.64	4.16	4.33	4.21
6	NK	2.02	3.03	3.63	3.73	3.92	3.79
Potassium							
1	Check	19.0	37.0	30.2	26.6	22.3	21.4
2	NPK	28.1	44.4	36.2	34.2	26.6	27.3
3	PK	22.1	35.2	29.6	29.4	24.2	23.8
4	P	23.9	41.8	31.0	30.2	25.5	24.5
5	N	22.6	38.7	31.4	29.1	25.8	25.1
6	NK	19.6	36.3	32.1	28.8	28.8	25.7

TABLE IV—(Continued)

PLOT	FERTILIZER TREATMENT	SUBSTANCES IN PLANT ON DATE OF SAMPLING					
		JUNE 25	JULY 6	JULY 16	JULY 24	JULY 31	AUG. 7
		mg.	mg.	mg.	mg.	mg.	mg.
		Soluble ash					
1	Check	38.4	74.2	61.8	58.9	53.1	54.7
2	NPK	55.5	89.7	74.0	76.8	63.9	69.2
3	PK	47.6	86.0	58.9	64.6	60.7	62.2
4	P	52.0	88.1	67.2	68.3	62.5	64.5
5	N	51.5	89.0	66.0	70.3	62.8	63.4
6	NK	42.9	73.1	66.7	65.2	66.7	62.6
		Ash					
1	Check	62.7	129	137	162	201	190
2	NPK	86.5	149	161	198	214	223
3	PK	79.5	141	148	187	212	213
4	P	76.3	148	156	191	206	228
5	N	78.7	132	141	173	218	216
6	NK	72.5	128	161	167	196	191

The data for the development of the whole plant are presented first. The results for all the plots are discussed collectively under a separate heading for each substance determined. After the data are presented from the standpoint of the plant as a whole, the distribution of the nutrients between heads and straw is discussed. The term straw is used to include all that portion of the plant, with the exception of the heads, lying above the ground level.

### Experimentation

#### PROGRESSIVE DEVELOPMENT OF THE WHOLE PLANT UPON APPLICATION OF VARIOUS NUTRIENTS

All the data for the average weights of substances per plant at the successive stages of growth (table IV), the corresponding percentage of these substances in dry matter (table V), and the amounts of substance in the whole plant at the various stages of growth as percentages of the maxima (table VI) are given in the following discussion.

#### DRY MATTER

The data for dry matter are presented graphically in figure 1. The average weight per plant on all plots except no. 6 increased from the first sampling to the last. The weights of plants on plot 6 reached a maximum the week before harvest and remained constant during the last week. Plots 2 and 4, whose fertilizer treatments were complete and phosphorus alone, respectively, yielded plants of greater weight through the entire growing

season. The plants on the check plot had approximately the same weight as those on plots 3, 5, and 6 for the first three samplings. Subsequently, the plants from the three fertilized plots gradually showed a slight increase over those from plot 1.

It is apparent that phosphorus had the most beneficial effect on growth of any of the three nutrients added. When potassium was added with

TABLE V

AVERAGE PERCENTAGE OF SUBSTANCES IN DRY MATTER OF ENTIRE AERIAL PORTION OF PLANT

PLOT	FERTILIZER TREATMENT	SUBSTANCES IN DRY MATTER ON DATE OF SAMPLING					
		JUNE 25	JULY 6	JULY 16	JULY 24	JULY 31	AUG. 7
		%	%	%	%	%	%
Phosphorus							
1	Check	0.273	0.235	0.183	0.174	0.175	0.190
2	NPK	0.282	0.231	0.177	0.178	0.166	0.174
3	PK	0.272	0.240	0.194	0.170	0.176	0.186
4	P	0.272	0.240	0.195	0.163	0.175	0.192
5	N	0.250	0.207	0.169	0.159	0.157	0.169
6	NK	0.259	0.211	0.158	0.169	0.162	0.163
Sulphur							
1	Check	0.289	0.271	0.216	0.207	0.191	0.194
2	NPK	0.261	0.239	0.184	0.179	0.169	0.168
3	PK	0.249	0.245	0.191	0.187	0.171	0.177
4	P	0.275	0.264	0.203	0.190	0.181	0.180
5	N	0.282	0.252	0.192	0.193	0.176	0.187
6	NK	0.266	0.242	0.183	0.191	0.183	0.195
Nitrogen							
1	Check	3.47	2.38	1.79	1.54	1.48	1.42
2	NPK	3.57	2.33	1.77	1.60	1.47	1.46
3	PK	3.36	2.31	1.76	1.55	1.48	1.42
4	P	3.40	2.32	1.77	1.59	1.47	1.43
5	N	3.73	2.55	1.82	1.61	1.49	1.46
6	NK	3.74	2.50	1.79	1.59	1.49	1.43
Calcium							
1	Check	0.460	0.336	0.243	0.221	0.216	0.202
2	NPK	0.443	0.331	0.227	0.235	0.210	0.206
3	PK	0.438	0.330	0.223	0.219	0.200	0.201
4	P	0.461	0.364	0.250	0.238	0.209	0.195
5	N	0.458	0.321	0.246	0.232	0.220	0.200
6	NK	0.468	0.303	0.239	0.217	0.197	0.192
Potassium							
1	Check	4.38	3.43	2.10	1.64	1.25	1.11
2	NPK	4.82	3.58	2.09	1.74	1.26	1.21
3	PK	4.24	3.26	1.96	1.62	1.19	1.16
4	P	4.25	3.29	1.90	1.57	1.19	1.07
5	N	4.46	3.62	2.12	1.63	1.31	1.19
6	NK	4.55	3.63	2.11	1.67	1.45	1.30

TABLE V—(Continued)

PLOT	FERTILIZER TREATMENT	SUBSTANCES IN DRY MATTER ON DATE OF SAMPLING					
		JUNE 25	JULY 6	JULY 16	JULY 24	JULY 31	AUG. 7
		%	%	%	%	%	%
Soluble ash							
1	Check	8.86	6.87	4.29	3.64	2.97	2.86
2	NPK	9.50	7.23	4.28	3.90	3.03	3.08
3	PK	9.14	7.96	3.90	3.55	2.99	3.02
4	P	9.25	6.94	4.12	3.56	2.91	2.83
5	N	10.2	8.24	4.46	3.93	3.19	3.00
6	NK	9.95	7.31	4.39	3.79	3.35	3.18
Ash							
1	Check	14.5	11.9	9.54	10.0	11.2	9.95
2	NPK	14.8	12.0	9.30	10.1	10.1	9.91
3	PK	15.3	14.0	9.82	10.3	10.4	10.3
4	P	13.6	11.7	9.54	9.95	9.58	10.0
5	N	15.5	12.3	9.56	9.66	11.1	10.2
6	NK	16.8	12.8	10.6	9.71	9.85	9.70

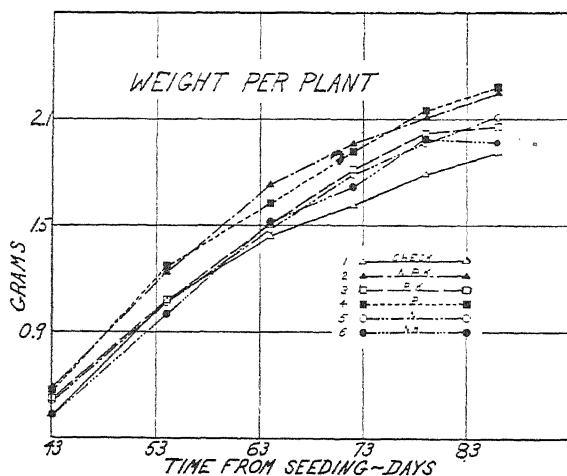


FIG. 1. Average weight per plant at successive dates of sampling.

phosphorus, the plants made a poorer growth than with phosphorus alone, but when a nitrate fertilizer was added in addition, as on plot 2, the deleterious effect of the potassium fertilizer was offset. It will be noted, however, that plot 4, treated only with treble superphosphate, supported just as heavy growth as plot 2, fertilized with all three nutrients.

Since the uptake of mineral nutrients takes place at a rapid pace early in the growth of the wheat plant, it might be expected that a readily available supply of all nutrients would favor a more rapid development of the



TABLE VI

AVERAGE AMOUNTS OF SUBSTANCES PRESENT IN AERIAL PORTION OF PLANT AT VARIOUS DATES  
OF SAMPLING EXPRESSED AS PERCENTAGES OF THE MAXIMA

PLOT	FERTILIZER TREATMENT	AV. AMOUNT OF SUBSTANCES ON DATES OF SAMPLING					
		JUNE 25	JULY 6	JULY 16	JULY 24	JULY 31	AUG. 7
		%	%	%	%	%	%
Dry matter							
1	Check	22.7	56.5	75.4	84.8	93.7	100.0
2	NPK	26.0	55.1	76.9	87.6	93.8	100.0
3	PK	25.3	52.4	73.3	88.3	98.5	100.0
4	P	24.6	55.7	71.5	84.2	94.3	100.0
5	N	24.0	50.7	70.1	84.8	93.4	100.0
6	NK	21.7	50.3	76.4	86.4	100.0	99.0
Phosphorus							
1	Check	32.0	68.8	71.5	76.4	85.1	100.0
2	NPK	42.2	73.1	78.3	89.5	89.5	100.0
3	PK	37.0	67.4	76.3	80.7	93.0	100.0
4	P	35.0	69.8	72.8	71.4	86.0	100.0
5	N	35.6	61.9	70.0	79.6	86.8	100.0
6	NK	34.8	65.5	74.5	90.4	100.0	100.0
Sulphur							
1	Check	33.8	79.2	84.1	90.5	92.4	100.0
2	NPK	40.2	78.3	84.1	93.4	94.4	100.0
3	PK	35.6	72.6	78.9	93.4	95.1	100.0
4	P	37.8	81.7	80.7	88.8	95.1	100.0
5	N	36.2	68.3	71.9	87.6	87.8	100.0
6	NK	29.9	62.9	72.2	85.2	94.8	100.0
Nitrogen							
1	Check	55.3	94.8	95.2	92.3	97.8	100.0
2	NPK	63.2	87.8	93.0	96.0	94.2	100.0
3	PK	58.1	82.7	88.4	93.7	100.0	97.3
4	P	58.6	90.5	88.7	93.9	96.9	100.0
5	N	61.2	88.3	87.4	93.2	95.1	100.0
6	NK	54.2	84.2	91.6	92.3	100.0	94.9
Calcium							
1	Check	51.4	93.8	90.4	92.5	100.0	99.7
2	NPK	55.9	88.6	84.9	100.0	95.7	99.8
3	PK	55.1	86.0	81.4	96.4	97.8	100.0
4	P	56.1	100.0	88.3	98.7	97.2	96.3
5	N	53.6	79.2	84.1	96.1	100.0	97.2
6	NK	51.5	77.3	92.6	95.2	100.0	96.7
Potassium							
1	Check	51.4	100.0	81.6	71.9	60.3	57.8
2	NPK	63.3	100.0	81.5	77.0	59.9	61.5
3	PK	62.8	100.0	84.1	83.5	68.8	67.6
4	P	57.2	100.0	74.2	72.2	61.0	58.6
5	N	58.4	100.0	81.1	75.2	66.7	64.9
6	NK	54.0	100.0	88.4	79.3	79.3	70.8

TABLE VI—(Continued)

PLOT	FERTILIZER TREATMENT	AV. AMOUNT OF SUBSTANCES ON DATES OF SAMPLING					
		JUNE 25	JULY 6	JULY 16	JULY 24	JULY 31	AUG. 7
		%	%	%	%	%	%
		Soluble ash					
1	Check	51.8	100.0	83.3	79.4	71.6	73.7
2	NPK	61.9	100.0	82.5	85.6	71.2	77.1
3	PK	55.3	100.0	68.5	75.1	70.6	72.3
4	P	59.0	100.0	76.3	77.5	70.9	73.2
5	N	57.9	100.0	74.3	79.0	70.6	71.2
6	NK	58.7	100.0	91.2	89.2	91.2	85.6
		Ash					
1	Check	31.2	64.2	68.2	80.6	100.0	94.5
2	NPK	38.8	66.8	72.2	88.8	96.0	100.0
3	PK	37.3	66.2	69.5	87.8	99.5	100.0
4	P	33.5	64.9	68.4	83.8	90.4	100.0
5	N	36.1	60.6	64.7	79.4	100.0	99.1
6	NK	37.0	65.3	82.1	85.2	100.0	97.4

plant during the early stages of growth. This is demonstrated in the case of plot 2 where the plants had a higher percentage of their maximum weight present at the first sampling than the plants of any of the other plots. The plants from plots 1 and 6 had a lower percentage of their maximum development present at the first sampling than the plants of the other plots.

#### PHOSPHORUS

The data for phosphorus are presented graphically in figure 2. The percentage of phosphorus of the plants of all plots showed a sharp decline to the third and fourth samplings when a slight increase took place. The plants from the three plots to which nitrate was added had the lowest percentages on the final sampling dates. The effect of phosphorus upon the percentage nitrogen is a well established fact and has been noted by many workers (1, 11), but the reciprocal relationship is not so well established. It must be observed, however, that at the first sampling the plants from plot 2 had the highest percentage phosphorus of the six, indicating that when ample phosphorus is available, nitrates do not exert this depressing effect during the early periods of rapid assimilation.

It was pointed out in the previous section that plot 3, fertilized with phosphorus and potassium, produced plants of lower weight than did plot 4, fertilized with phosphate alone. That this deleterious effect was not due to a depressing effect of potassium upon phosphorus assimilation is shown by the percentage data in figure 2. The percentages of phosphorus in the plants from plots 3 and 4 were nearly identical from the first sampling to the last, and both were comparatively high.

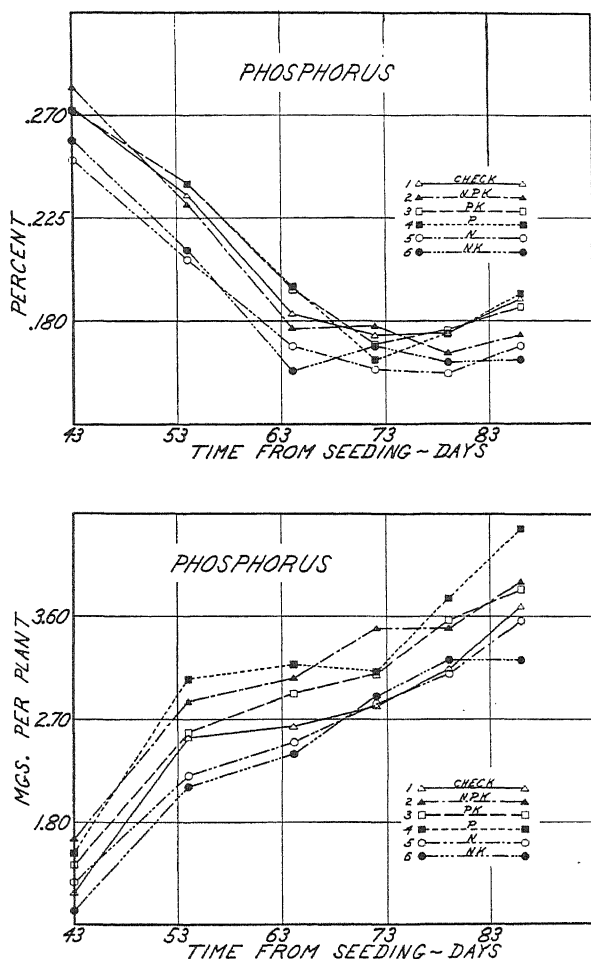


FIG. 2. Upper graph, phosphorus as percentage of dry matter; lower graph, phosphorus as milligrams per plant.

In all cases the maximum amount of phosphorus was present in the plants at the last sampling and the plants from all plots, except plot 6, showed a considerable increase between the last two samplings. This is at variance with the findings of both the German and the English workers.

#### SULPHUR

The data for sulphur are presented graphically in figure 3. Between the first and second samplings the percentage of sulphur of the plants from all the plots showed a slight decrease. Between the second and third samplings the plants from all the plots again followed similar trends and

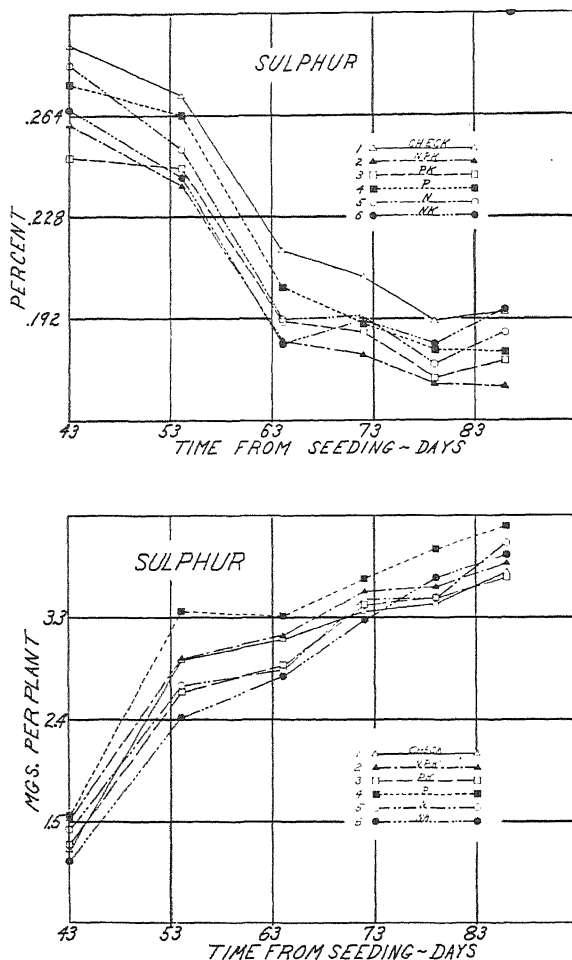


FIG. 3. Upper graph, sulphur as percentage of dry matter; lower graph, sulphur as milligrams per plant.

decreased sharply. With one or two exceptions there was once more a slow decrease in the percentage of sulphur from the third to sixth samplings. The plants from the check plot showed the highest percentage of sulphur throughout their entire growth, indicating that an ample supply of sulphur is present in the soil and that in the check plot sulphur took the place of some of the plant nutrients that were added to the fertilized plots.

This disposition of plants to take up readily available minerals is again demonstrated in the case of the completely fertilized plot 2. At four of the six sampling dates the plants from plot 2 had the lowest percentage of sulphur, showing that in the presence of an ample supply of phosphorus,

potassium, and nitrogen sulphur was used less extensively. Although the plants on plots 2 and 4 made nearly identical growth, the percentage of sulphur in the plants of plot 4 was at least 10 per cent. greater than that in the plants of plot 2 at all stages of growth.

The milligrams of sulphur per plant increased progressively in all plots during the entire growing season. Most of the sulphur, however, had been assimilated by the plant at the time of the second sampling. The percentages of the maxima at this time ran from 62.9 per cent. in the case of plot 6 to 81.7 per cent. in the case of plot 4.

No comparisons can be made with the English or the German investigations for neither study included a determination of sulphur. WOODFORD and McCALLA (16) determined sulphur and found it increasing in the plants from the two plots grown on black soil until next to the last sampling, when a sharp decrease took place. The amounts of sulphur in the plants from the plots grown on the gray soil showed a slight increase from the first to the fourth samplings and remained practically constant from that time until harvest.

The data for sulphur apparently reflect the effect of the very dry weather and of the subsequent irrigation. The rate of assimilation diminished between the second and third samplings and increased again after water was finally applied to the plots.

#### NITROGEN

The graphical representation of the nitrogen data is given in figure 4. The percentage of nitrogen decreased asymptotically from the first to the last samplings. The percentages on the last four samplings are extremely close together. In fact on next to the last sampling date the percentage data from all six plots are very nearly within the limits of the experimental error of a Kjeldahl nitrogen determination.

These data demonstrate the effectiveness of a progressive development study in plant nutrition for it was only at the first two sampling dates that the effect of nitrates upon the percentage composition of the plant could be observed. At the first sampling the plants from plots 5 and 6, to which nitrate, and nitrate plus potassium were added, respectively, had the highest percentage of nitrogen and the next highest percentage was found in the plants of plot 2, fertilized with nitrate, potassium, and phosphorus.

When the amounts of nitrogen are plotted, wide differences become apparent between the various plots. The plants from plots 2 and 4 showed the highest amount of nitrogen at all sampling dates, with the plants from plot 2 running considerably above those from plot 4 at the third and fourth samplings. This may possibly be due to the fact that nitrate was added to plot 2 and hence was available during the dry period when the production of nitrates by soil bacteria had probably almost ceased.

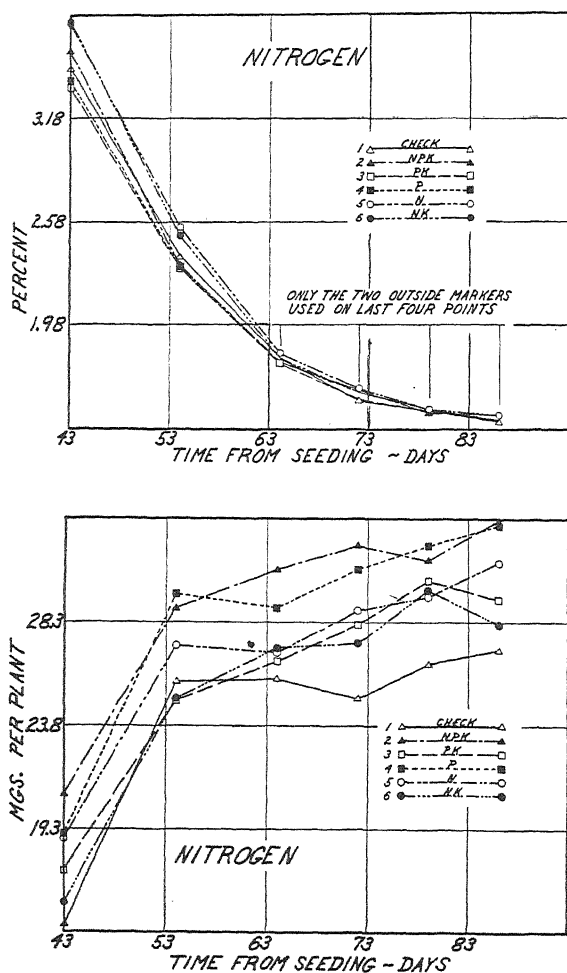


FIG. 4. Upper graph, nitrogen as percentage of dry matter; lower graph, nitrogen as milligrams per plant.

A large amount of nitrogen was assimilated by the wheat on all plots between the first and second samplings and a much smaller amount entered the plant from the second sampling until harvest. The amounts of nitrogen present at the second sampling as percentages of the maxima run from 82.7 to 94.8 per cent. However, the data show nitrogen increased until the final harvest on all plots except plots 3 and 6. These two plots showed a slight decrease between the fifth and sixth samplings.

The German investigation showed nitrogen reached a maximum three weeks before harvest and suffered a subsequent loss of 20 per cent. KNOWLES and WATKIN (9) found that nitrogen reached a maximum about four weeks

before harvest, but remained constant for the remainder of the growing season. BRENCHELY (4) found assimilation of nitrogen proceeding until a week before maturity was reached. The investigation of WOODFORD and MCCALLA (16) shows nitrogen increasing until the final harvest.

### CALCIUM

The data for calcium are presented graphically in figure 5. Little information can be drawn from the percentage data of the calcium determinations. The percentages of calcium in the plants from all the plots at the various dates of sampling are very similar. The percentage of cal-

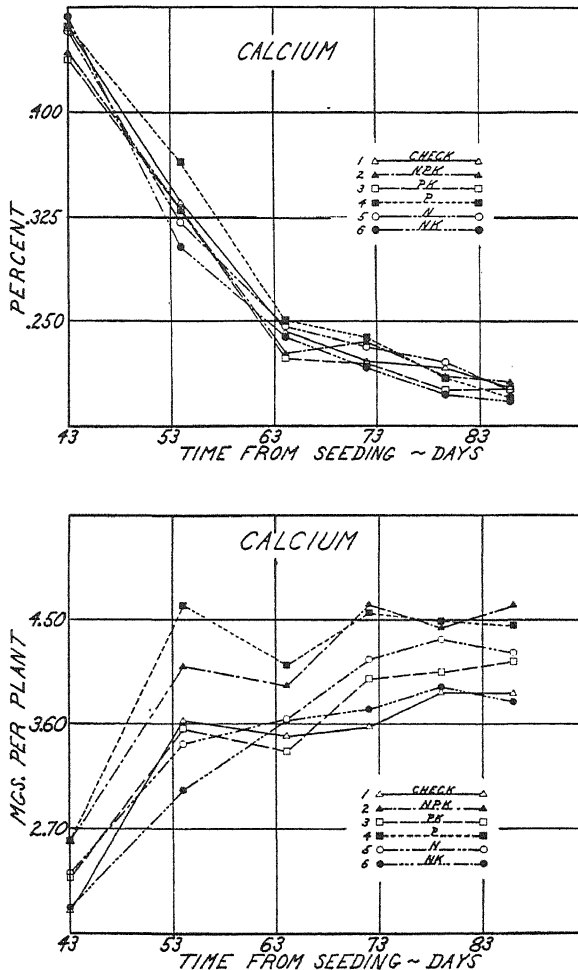


FIG. 5. Upper graph, calcium as percentage of dry matter; lower graph, calcium as milligrams per plant.

cium decreased in all cases from the first to the last sampling with by far the largest decrease taking place between the first and third samplings.

The data for the milligrams of calcium per plant show a heavy assimilation took place between the first and second samplings. With the exception of the plants from plots 5 and 6, there was a loss of calcium between the second and third samplings. Between the third and fourth samplings a reversal of the movement of calcium took place and there once more is a gain. From the fourth to the last samplings little change takes place. This reversal of movement of calcium was without doubt the effect of the hot dry weather upon the plant. When water was applied to the plants assimilation increased and there was a net gain in the amount of calcium in the plant.

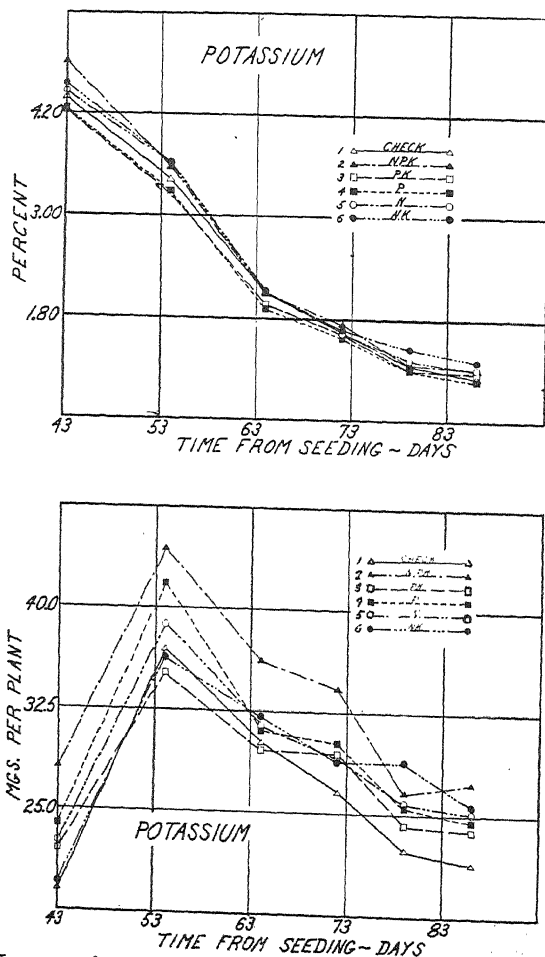


Fig. 6. Upper graph, potassium as percentage of dry matter; lower graph, potassium as milligrams per plant.



Calcium was not determined in the German investigation, but the English workers reported a loss of 19 per cent. of the calcium during the last six weeks of growth. The results of WOODFORD and MCCALLA (16) show a loss of calcium during the last fifteen days in the plants from two of their four plots. A small loss was noted in the present investigation in the case of the plants from plots 4, 5, and 6. The plants from plot 4 showed the largest amount of calcium at the second sampling, the plants from plots 1, 5, and 6 at the fifth sampling, and the plants from plot 3 at the last sampling.

### POTASSIUM

The data for potassium are presented graphically in figure 6. The percentage of potassium in the plants from all plots decreased steadily from the first sampling until harvest with the plants from each plot showing a uniform decrease. The difference between the plants of the various plots was not pronounced but it will be noted that in all cases the plants from plots to which nitrate was added have the highest percentage of potassium.

The plants from plot 3, which was fertilized with phosphorus and potassium, and from plot 4, which was fertilized with phosphorus alone, had the lowest percentage of potassium at all stages of growth. The plants from plot 2, which received an application of nitrate in addition to the phosphorus and potassium, contained a higher percentage of potassium. These facts indicate that phosphorus depresses the assimilation of potassium unless there is an ample supply of nitrates available to the plant.

The total amount of potassium in the plants of all plots reached a maximum at the second sampling and decreased from that time until harvest. The amounts of potassium lost by the plants, expressed as percentages of the maxima, vary from 29.2 per cent. in the plants of plot 6 to 42.2 per cent. in the plants from plot 1. The rapid assimilation of potassium and the attainment of a maximum early in the growth of the wheat plant was observed by the German workers, the English workers, and WOODFORD and MCCALLA (16). BERRY (3) found the same thing in his study of the progressive development of the oat plant.

### SOLUBLE ASH

The data for soluble ash are presented graphically in figure 7. The curves plotted for the percentage of soluble ash in the plants are nearly identical with the curves for the percentage of potassium in the plant. This is not strange when it is observed that the weights of potassium in the plants are nearly one-half the weights of soluble ash. When the weights of the anions accompanying the potassium are taken into consideration, it is evident that potassium salts must compose the major portion of the soluble ash.

The wheat from the three plots to which nitrates were added had the

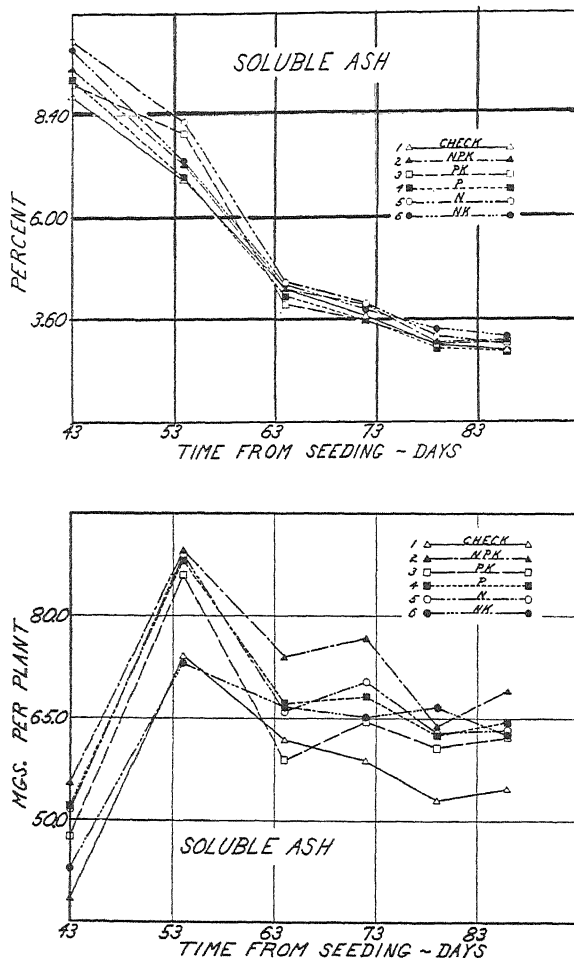


FIG. 7. Upper graph, soluble ash as percentage of dry matter; lower graph, soluble ash as milligrams per plant.

highest percentages of soluble ash. The wheat from these three plots also had the highest percentage of potassium.

The amount of soluble ash in the plants again reflects the behavior of potassium. Like potassium, the soluble ash reached a maximum in the plants from all the plots at the second sampling. From the second to the third sampling a considerable loss occurred, but in the interim between the third and fourth samplings the loss was not so large in the plants from plots 4, 5, and 6 and there was even a gain in the plants from plots 2, 3, and 4. This gain in soluble ash was probably due to the combined effect of the decrease in the downward movement of potassium and the increase in the assimilation of phosphorus, sulphur, and calcium.

## ASH

The data for ash are presented graphically in figure 8. The percentage of ash declined until the third sampling and then showed a slight increase

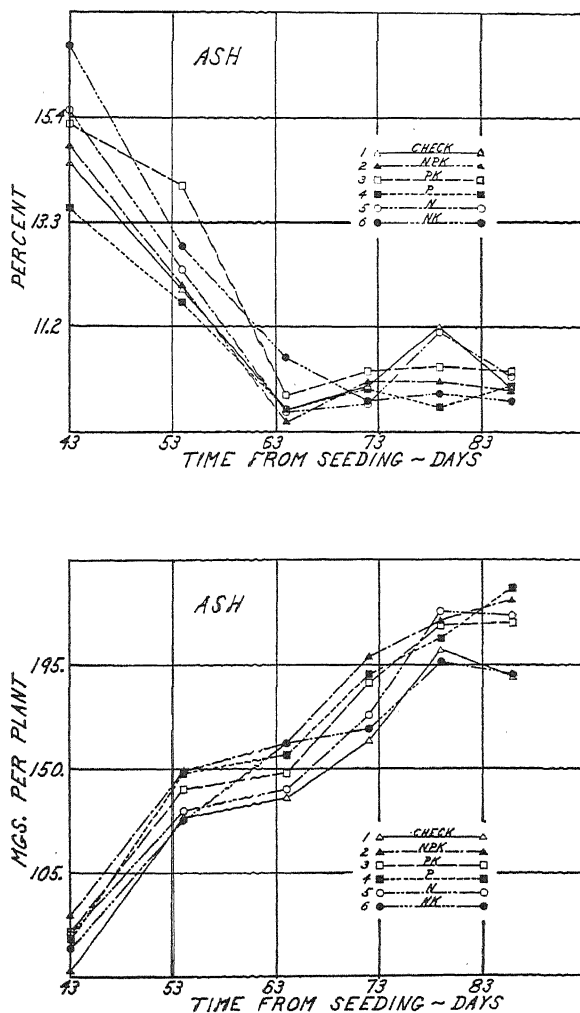


FIG. 8. Upper graph, ash as percentage of dry matter; lower graph, ash as milligrams per plant.

in most cases. An unusual jump in percentage of ash occurred in the plants from plots 2 and 5 at the fifth sampling date. The writer can advance no logical explanation for this unless it may be attributed to the error which would be reflected in the amount of ash by the inclusion of soil or dust particles in the samples. Care was taken to remove as much of the adhering

soil from the plants as possible, but it may be that some soil escaped detection. Any soil left adhering to the plants would, of course, add to the amount of ash determined.

The amounts of ash in the plants from all plots increased from the first to the fifth samplings. In the period between the last two samplings the amount of ash in the plants from plots 2, 3, 5, and 6 remained fairly constant while the ash in the plants from plot 4 continued to increase.

#### EFFECTS OF NUTRIENTS ON THE WHOLE PLANT

The results have shown that the wheat grown for this investigation was still assimilating most of the plant nutrients at the time of harvest. Potassium reached a maximum at the second sampling date and suffered a subsequent loss, but sulphur, phosphorus, and nitrogen were still entering the plant when the last samples were collected. The amounts of calcium changed very little during the last two weeks of growth.

The previous studies on the progressive development of the wheat plant have shown assimilation ceasing earlier in the growth of the plant. The German workers (15) found nitrogen and phosphorus reached their maxima three weeks before harvest. Nitrogen suffered a subsequent loss and phosphorus remained constant. The English workers (9) found nitrogen and phosphorus assimilation ceased several weeks before harvest and the amount remained practically constant until harvest. They did not determine sulphur, but found calcium reached a maximum slightly in advance of phosphorus and nitrogen and later suffered a slight loss. WOODFORD and McCALLA (16) found nitrogen increased until harvest. In three of their four plots phosphorus increased until harvest. Calcium showed but little change during the last three weeks. Sulphur reached a maximum two weeks before harvest and suffered a subsequent loss in two of their four plots. In the other two little change took place in the last three weeks.

The probable cause of this late assimilation of plant nutrients is the relatively short period of time which elapsed between seeding and harvesting of the wheat used in this study. The wheat was seeded on May 13 and harvested 87 days later on August 8. The average time between the seeding and harvesting of spring wheat in Montana is close to 120 days (2). The wheat used in the German study was seeded April 23 and harvested 127 days later on August 28. The period for the wheat used in the Canadian study was 114 days. It is apparent that an 87-day period is much shorter than the normal period of growth.

From the data of the three investigations in which spring wheat was grown, it is obvious that the length of the growing season may alter the conclusions reached in a study of this kind. The evidence indicates that the plant tends to assimilate nutrients over a certain length of time re-

ardless, or, perhaps we should say, in spite of its state of desiccation. Naturally the amount of moisture in a plant may in a large measure govern the movement of nutrients, but it is probably true that if the growing season of the wheat plant has been cut abnormally short by drought, nutrients will continue to be assimilated until late in the life of the plant.

As described earlier in this paper, the wheat was irrigated on July 21, three days before the fourth set of samples was collected. The effect of this irrigation upon the movement of nutrients in the wheat plant is definitely shown by the shape of the curves representing the milligrams of nutrients per plant. From July 6 to 16 there was a definite reduction in the rate of assimilation of nearly all the nutrients, indicating that the plants were drying out to such an extent that movement of nutrients was becoming extremely slow. The data for the last three samplings show the effect of the irrigation on July 21. It is evident that the roots of the plant were able to absorb enough moisture to allow the nutrients to once more resume their movement in the plant.

This is illustrated nicely by the movement out of the plant by potassium. Between the second and third samplings potassium moved out rapidly, but the rate decreased markedly between the third and fourth samplings. After the water was applied to the plots, the downward movement was again accelerated. Although the decrease in the movement of potassium was not found in the plants from plots 1, 5, and 6, it was very apparent in the plants from the remaining plots. It is interesting to note that in the case of all the nutrients determined, the added moisture resulting from the irrigation tended to produce an upward movement into the plant while in the case of potassium the downward movement was accelerated. Thus it appears that the downward movement of potassium is due in part to factors other than the natural desiccation of the plant as it approaches maturity.

The anions which are associated with potassium in its downward migration are not easily identified in this study. It is quite possible that some sulphate ions migrate from the upper portion of the plant with the potassium. As mentioned before, WOODFORD and MCCALLA (16) found a loss of sulphur occurred in the wheat from two of their plots. The English workers were the only ones to include a determination of chlorine and they found that chlorine moved downward in the plant at about the same time and at the same rate as did potassium. The loss in equivalents of chlorine, however, was far less than the loss in equivalents of potassium. It is possible, therefore, that the gain or loss noted in the nutrients of the wheat plant over a period of time is a net gain or loss resulting from the movement of ions both upward and downward in the plant.

The plants from plots 5 and 6, which were fertilized with nitrogen and nitrogen and potassium, respectively, exhibited a behavior decidedly

different from the plants of the other plots. This is especially true in the case of the plants of plot 6. It will be noted that the movement of most of the nutrients in the plants of these plots was less affected by the drought and subsequent irrigation. It has been frequently observed that an ample supply

TABLE VII

AMOUNTS OF SUBSTANCES IN LEAVES AND STEMS, AND IN HEADS, EXPRESSED  
AS MILLIGRAMS PER PLANT

PLOT	FERTILIZER TREATMENT	SUBSTANCES ON DATE OF SAMPLING					
		LEAVES AND STEMS			HEADS		
		JULY 24	JULY 31	AUG. 7	JULY 24	JULY 31	AUG. 7
		mg.	mg.	mg.	mg.	mg.	mg.
Dry matter							
1	Check	1130	1010	950	490	780	960
2	NPK	1320	1170	1180	650	940	1070
3	PK	1180	1080	1040	640	950	1020
4	P	1240	1180	1130	680	970	1150
5	N	1220	1150	1080	570	820	1030
6	NK	1130	1120	1000	590	870	970
Phosphorus							
1	Check	1.06	0.61	0.47	1.76	2.53	3.22
2	NPK	1.19	0.69	0.55	2.31	2.81	3.36
3	PK	1.04	0.67	0.52	2.06	2.90	3.32
4	P	1.00	0.77	0.53	2.12	2.99	3.84
5	N	0.90	0.67	0.51	1.94	2.43	3.06
6	NK	0.88	0.65	0.45	2.03	2.57	2.77
Sulphur							
1	Check	2.62	1.92	1.88	0.73	1.50	1.82
2	NPK	2.68	1.91	1.92	0.85	1.66	1.86
3	PK	2.54	1.87	1.78	0.87	1.60	1.87
4	P	2.68	2.22	2.06	0.96	1.68	2.04
5	N	2.64	2.09	2.01	0.82	1.38	1.94
6	NK	2.42	2.10	1.94	0.86	1.55	1.91
Nitrogen							
1	Check	13.8	8.59	6.09	11.2	17.9	21.0
2	NPK	16.1	9.59	7.79	15.5	21.4	25.1
3	PK	13.7	9.07	6.66	14.5	21.0	22.6
4	P	14.8	10.3	7.12	15.8	21.3	25.5
5	N	15.3	10.9	7.67	13.5	18.5	23.2
6	NK	13.3	10.4	6.90	14.1	19.3	21.3
Calcium							
1	Check	3.10	3.09	3.07	0.48	0.78	0.79
2	NPK	4.00	3.53	3.66	0.63	0.90	0.96
3	PK	3.35	3.20	3.28	0.64	0.85	0.86
4	P	3.86	3.65	3.48	0.70	0.84	0.97
5	N	3.57	3.58	3.33	0.59	0.75	0.88
6	NK	3.19	3.12	2.95	0.54	0.80	0.84

TABLE VII—(Continued)

PLOT	FERTILIZER TREATMENT	SUBSTANCES ON DATE OF SAMPLING					
		LEAVES AND STEMS			HEADS		
		JULY 24	JULY 31	AUG. 7	JULY 24	JULY 31	AUG. 7
		mg.	mg.	mg.	mg.	mg.	mg.
Potassium							
1	Check	22.3	16.2	14.3	4.32	6.10	7.09
2	NPK	28.2	18.8	19.2	6.03	7.76	8.08
3	PK	23.2	17.0	16.0	6.16	7.24	7.77
4	P	23.6	17.7	16.4	6.58	7.76	8.13
5	N	23.9	19.0	17.1	5.16	6.84	7.98
6	NK	23.3	20.8	17.8	5.50	8.02	7.92
Soluble ash							
1	Check	46.3	34.5	32.8	12.6	18.6	21.9
2	NPK	60.2	41.4	45.1	16.6	22.5	24.1
3	PK	48.9	38.7	39.1	15.7	22.0	23.1
4	P	50.6	40.0	39.0	17.7	22.5	25.5
5	N	55.8	43.5	40.0	14.5	19.3	23.4
6	NK	49.9	45.1	40.0	15.3	21.6	22.6
Ash							
1	Check	134	155	135	28.2	46.0	55.4
2	NPK	163	162	164	35.0	51.7	58.7
3	PK	152	158	155	34.9	54.1	57.5
4	P	153	152	161	38.0	53.7	66.6
5	N	142	171	159	31.1	47.4	57.3
6	NK	136	148	138	31.4	47.8	53.4

of nitrates produces heavy vegetative growth and tends to delay maturity (13, 14). The plants on these plots did not appear to have excessive leaf growth, but they did possess a high percentage of soluble salts. It may be that this fact contributed to their being less affected by the drought as regards the movement of nutrients.

#### DISTRIBUTION OF SUBSTANCES BETWEEN HEADS AND STRAW

The samples gathered on the last three sampling dates were divided into heads and straw for separate analyses. Table VII gives the amounts of substances, expressed as milligrams per plant, present in the heads and in the straw. Table VIII gives the amounts of substances present in the straw and in the heads, expressed as percentages of the dry matter, Table IX gives the amounts of substances present in the heads at the last three dates of sampling, expressed as percentages of the amounts present in the whole plant.

Figures 9 and 10 represent graphically the milligrams of substances present in the whole plant at the last three samplings and the distribution of these substances between the heads and the straw. The data from the three

successive samplings are grouped together for each plot. The six groups representing the six plots are arranged in numerical order from left to right. All eight charts in figures 9 and 10 are drawn to the same scale with the appropriate factor given with the name of the substance to illustrate the comparative amounts of the different substances present in the plant.

TABLE VIII

AVERAGE PERCENTAGE OF SUBSTANCES IN DRY MATTER OF LEAVES AND STEMS, AND OF HEADS

PLOT	FERTILIZER TREATMENT	SUBSTANCES IN DRY MATTER ON DATE OF SAMPLING					
		LEAVES AND STEMS			HEADS		
		JULY 24	JULY 31	AUG. 7	JULY 24	JULY 31	AUG. 7
		%	%	%	%	%	%
Phosphorus							
1	Check	0.094	0.060	0.049	0.360	0.324	0.328
2	NPK	0.090	0.059	0.047	0.355	0.299	0.314
3	PK	0.088	0.062	0.050	0.322	0.305	0.325
4	P	0.081	0.065	0.047	0.312	0.308	0.334
5	N	0.074	0.058	0.047	0.340	0.296	0.297
6	NK	0.078	0.058	0.045	0.344	0.295	0.286
Sulphur							
1	Check	0.232	0.190	0.198	0.148	0.192	0.190
2	NPK	0.203	0.163	0.163	0.131	0.177	0.174
3	PK	0.215	0.173	0.171	0.136	0.168	0.183
4	P	0.216	0.188	0.182	0.141	0.173	0.177
5	N	0.216	0.182	0.186	0.143	0.168	0.188
6	NK	0.214	0.188	0.194	0.145	0.178	0.197
Nitrogen							
1	Check	1.22	0.85	0.64	2.29	2.30	2.19
2	NPK	1.22	0.82	0.66	2.39	2.28	2.35
3	PK	1.16	0.84	0.64	2.26	2.21	2.22
4	P	1.19	0.87	0.63	2.33	2.20	2.22
5	N	1.25	0.95	0.71	2.37	2.26	2.25
6	NK	1.18	0.93	0.69	2.39	2.22	2.20
Calcium							
1	Check	0.275	0.306	0.323	0.097	0.092	0.082
2	NPK	0.303	0.298	0.310	0.097	0.100	0.090
3	PK	0.284	0.296	0.315	0.100	0.089	0.084
4	P	0.311	0.309	0.308	0.103	0.087	0.084
5	N	0.293	0.311	0.308	0.103	0.092	0.085
6	NK	0.282	0.279	0.295	0.092	0.092	0.087
Potassium							
1	Check	1.97	1.60	1.50	0.882	0.782	0.739
2	NPK	2.14	1.61	1.63	0.927	0.825	0.755
3	PK	1.97	1.57	1.54	0.963	0.762	0.762
4	P	1.90	1.50	1.45	0.967	0.800	0.707
5	N	1.96	1.65	1.58	0.905	0.834	0.775
6	NK	2.06	1.86	1.78	0.933	0.922	0.817



TABLE VIII—(Continued)

PLOT	FERTILIZER TREATMENT	SUBSTANCES IN DRY MATTER ON DATE OF SAMPLING					
		LEAVES AND STEMS			HEADS		
		JULY 24	JULY 31	AUG. 7	JULY 24	JULY 31	AUG. 7
		%	%	%	%	%	%
		Soluble ash					
1	Check	4.10	3.42	3.45	2.58	2.38	2.28
2	NPK	4.56	3.54	3.82	2.56	2.39	2.25
3	PK	4.14	3.58	3.76	2.46	2.32	2.26
4	P	4.08	3.39	3.45	2.61	2.32	2.22
5	N	4.57	3.78	3.70	2.54	2.35	2.27
6	NK	4.42	4.03	4.00	2.60	2.48	2.33
		Ash					
1	Check	11.9	15.3	14.2	5.76	5.89	5.77
2	NPK	12.4	13.8	13.9	5.38	5.50	5.49
3	PK	12.9	14.7	14.9	5.46	5.69	5.64
4	P	12.3	12.9	14.3	5.59	5.54	5.79
5	N	11.7	14.8	14.7	5.46	5.78	5.56
6	NK	12.0	13.2	13.8	5.32	5.49	5.51

The percentages of substances present in the heads of the plants were in most cases quite different from the percentages of substances in the whole plant. It may be noted, however, that if a fertilizer treatment affected the

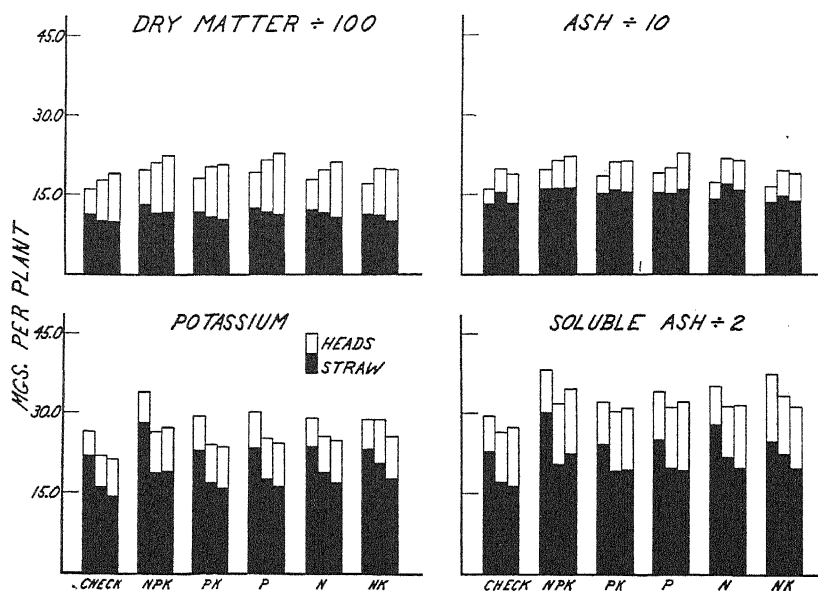


FIG. 9. Amounts of substances (expressed as milligrams per plant) present in the whole plant on the last three sampling dates and distribution of these substances between straw and heads.

TABLE IX

AVERAGE AMOUNTS OF SUBSTANCES IN HEADS OF PLANT EXPRESSED AS PERCENTAGES OF  
AMOUNTS PRESENT IN ENTIRE AERIAL PORTION

PLOT	FERTILIZER TREATMENT	AV. AMOUNTS OF SUBSTANCES ON DATE OF SAMPLING		
		JULY 24	JULY 31	AUGUST 7
		%	%	%
		Dry matter		
1	Check	30.2	43.6	50.3
2	NPK	33.0	44.5	47.6
3	PK	35.2	46.8	49.5
4	P	35.4	45.1	50.4
5	N	31.8	41.6	48.8
6	NK	34.3	43.7	49.2
		Phosphorus		
1	Check	62.4	80.6	87.3
2	NPK	66.0	80.3	85.9
3	PK	66.5	81.2	86.5
4	P	67.9	79.5	87.9
5	N	68.3	78.4	85.7
6	NK	69.8	79.8	86.0
		Sulphur		
1	Check	21.8	43.8	49.2
2	NPK	24.1	46.5	49.2
3	PK	25.5	46.1	51.2
4	P	26.4	43.1	49.8
5	N	23.7	39.8	49.1
6	NK	26.2	42.5	49.6
		Nitrogen		
1	Check	44.8	67.5	77.5
2	NPK	49.1	69.0	76.3
3	PK	51.4	69.8	77.1
4	P	51.6	67.4	78.2
5	N	46.9	62.9	75.1
6	NK	51.5	65.0	75.5
		Calcium		
1	Check	13.4	20.2	20.5
2	NPK	13.6	20.3	20.8
3	PK	16.0	21.0	20.8
4	P	15.4	18.7	21.8
5	N	14.2	17.3	20.9
6	NK	14.5	20.4	22.2
		Potassium		
1	Check	16.2	27.4	33.1
2	NPK	17.6	29.2	29.6
3	PK	21.0	29.9	32.6
4	P	21.8	30.4	33.2
5	N	17.7	26.5	31.8
6	NK	19.1	27.8	30.8

TABLE IX—(Continued)

PLOT	FERTILIZER TREATMENT	AV. AMOUNTS OF SUBSTANCES ON DATE OF SAMPLING		
		JULY 24	JULY 31	AUGUST 7
		%	%	%
		Soluble ash		
1	Check	21.3	35.0	40.0
2	NPK	21.6	35.2	34.8
3	PK	24.3	36.2	37.1
4	P	25.9	36.0	39.5
5	N	20.6	30.7	36.9
6	NK	23.5	32.4	36.1
		Ash		
1	Check	17.4	22.9	29.2
2	NPK	17.7	24.2	26.3
3	PK	18.7	25.5	27.0
4	P	19.9	26.1	29.2
5	N	18.0	21.7	26.5
6	NK	18.8	24.4	28.0

composition of the whole plant in a certain manner, it also affected the composition of the head similarly. For example, the lowest percentage of sulphur was found in the plants from plot 2, and likewise the heads from plot 2 had the lowest percentage of sulphur.

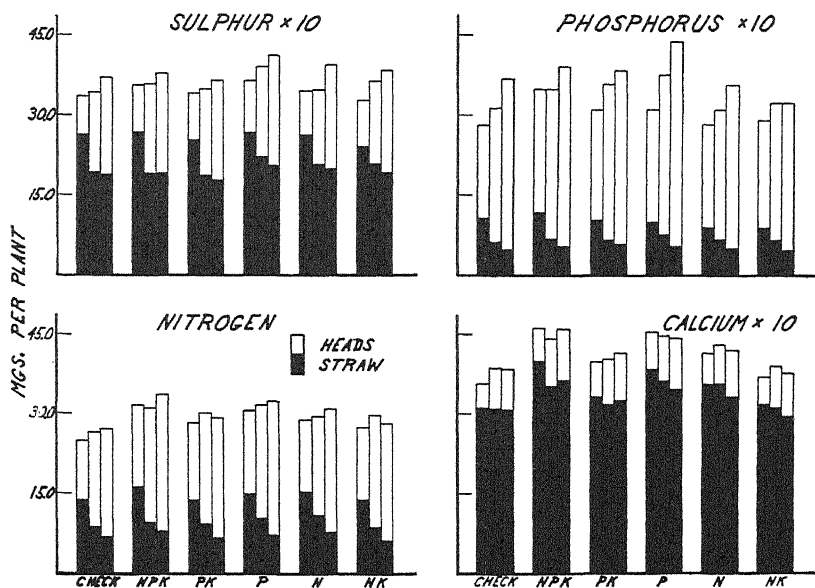


FIG. 10. Amounts of substances (expressed as milligrams per plant) present in the whole plant on the last three sampling dates and distribution of these substances between straw and heads.

The only exception found to this observation is the case of nitrogen. The percentage of nitrogen in the heads of plants from plot 2 was considerably higher than the percentage of nitrogen in the heads of the plants from the other plots in spite of the fact that the whole plants from all of the plots had nearly identical nitrogen percentages at the last three sampling dates.

At the last two sampling dates the straw of plots 5 and 6 had higher percentages of nitrogen than the straw from the remaining plots. The reason for this fact is seen in the values given in table IX, which show that a lower percentage of the nitrogen of the whole plant was transferred to the heads of the plants from plots 5 and 6.

There was remarkably little difference between the relative weights of heads per plant from the various plots. On all plots the heads formed almost exactly half the weight of the whole plant at harvest and it appears that the type of fertilizer applied to the plot had little effect upon this ratio.

Figures 9 and 10 show that there were approximately the same amounts of sulphur, phosphorus, and calcium present in the whole plant at maturity but that the distribution of these elements between the heads and the straw varied markedly. Approximately 85 per cent. of the phosphorus present in that portion of the plant lying above the ground was found in the heads. The heads contained approximately 50 per cent. of the sulphur and only 20 per cent. of the calcium. The elements nitrogen and potassium each represented about the same percentage of the dry matter of the mature plant. This percentage is approximately eight times the percentage of any of the three elements, sulphur, phosphorus, or calcium, and, although nitrogen and potassium were present in the whole plant in approximately equal amounts, a large difference was found in the way they were distributed between the heads and the remainder of the plant. The heads contained about 75 per cent. of the nitrogen, but only 30 per cent. of the potassium. A greater proportion of phosphorus was found in the wheat heads than any of the five elements studied.

In nearly all cases the amounts of all the substances in the heads of the plants increased from the fourth to the sixth samplings. The rate of increase was very rapid between the fourth and fifth samplings and somewhat slower between the fifth and sixth samplings. Practically all the calcium and most of the potassium which entered the heads was found there at the fifth sampling. On the other hand, phosphorus, sulphur, and nitrogen were still entering the heads at the final sampling date.

### Summary

1. The nutrition of Marquis wheat as influenced by five different fertilizer treatments was followed progressively through the growing season.
2. With few exceptions the amounts of dry matter, phosphorus, sulphur,

and nitrogen increased progressively from the first to the last sampling. Potassium reached a maximum in the plants from all plots one month before harvest and suffered a subsequent loss from that time until harvest. Calcium increased rapidly until one month before harvest and then remained fairly constant.

3. Curves for the rate of assimilation of the nutrients showed the effect of drought and subsequent irrigation upon the movement of plant nutrients. As the desiccation progressed there was a decreased movement of phosphorus, calcium, nitrogen, and sulphur into the plant and following the application of water the upward movement again increased. Potassium was moving out of the plant at this time and the desiccation of the plant decreased the downward movement. Following irrigation the downward movement increased.

4. The results show that the length of the growing season may alter the conclusions drawn from a progressive development study.

5. On all the plots the heads formed almost exactly half the dry weight of the aerial portion of the plant and the type of fertilizer treatment had little effect upon this ratio.

6. Approximately equal amounts of calcium, phosphorus, and sulphur were present in the plant at maturity, but the distribution of these elements between heads and straw varied markedly.

7. Practically all the calcium and most of the potassium which entered the heads was found there a week before harvest. Phosphorus, sulphur, and nitrogen, however, were still entering the heads when the grain was harvested.

DEPARTMENT OF CHEMISTRY

MONTANA AGRICULTURAL EXPERIMENT STATION

BOZEMAN, MONTANA

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# SALT TOLERANCE OF PLANTS AT VARIOUS TEMPERATURES<sup>1</sup>

S. M. AHI AND W. L. POWERS

(WITH THREE FIGURES)

## Introduction

The interesting observation was made that yellow sage (*Chrysothamnus viscidiflorus*) grows in Klamath Basin at some 4200 feet elevation on soil having a reaction of pH 9.5 to 10, while in the hotter climate of Malheur Valley, at an elevation of 2000 feet, it grows on soil of a reaction value of approximately 9. Small grain also appears to be more resistant to alkali at the cooler, more elevated alkaline areas. EATON (7) has also suggested that temperature affects salt tolerance. Experiments were undertaken as a phase of a salt tolerance study to determine the effect of temperature on salt tolerance of certain plants, the degree to which certain plants differ in salt tolerance, the effect of chemical composition of the alkaline soils, and of other factors on salt tolerance.

## Historical review

Reclamation and use of alkaline land is one of the major soil problems of arid agriculture. HARRIS (16) estimated that some 13 per cent. of the irrigated land contained sufficient alkali<sup>2</sup> to be seriously harmful to plants.

## EVOLUTION AND CLASSIFICATION OF ALKALI SOILS

Any soluble salt that is present in the soil in excess may be called alkali. HILGARD (22) distinguished two types of alkali: (a) "white alkali" which includes one or more of such salts as sodium chloride, sodium sulphate, sodium nitrate, magnesium sulphate, magnesium chloride, calcium chloride; (b) "black" alkali, which usually consists mainly of sodium carbonate. "Black" alkali is more destructive to plants than "white" alkali.

GLINKA (12) classifies soils into the three following groups: (a) solonchak, (b) solonetz, and (c) solod. According to GEDROIZ (8) these three subgroups represent three consecutive stages of the process which may be called "evolution of alkali soil."

Such an evolution consists of three consecutive transformations: Salinization, which transforms an original soil into solonchak; solonization, transforming solonchak into solonetz; and solotization, which transforms solonetz to the final product of the evolutionary process, solod (or soloth). Sol-

<sup>1</sup> Published as technical paper no. 275 with the approval of the Director as a contribution from the Soils Department of the Oregon Agricultural Experiment Station.

<sup>2</sup> "Alkali" land is here used as a popular term to refer to soil that is saline, or alkaline, or both.

onization, according to NIKIFOROFF (27), involves a desalinization with a partial substitution of monovalent exchangeable cations, especially sodium for calcium. Solotization involves hydrolysis and disintegration of the colloidal complex with a high degree of saturation of sodium, and a deflocculated state with soluble salts washed out and a columnar structure usually in the B horizon. The final product, solod, has reduced exchange capacity with the cations partly substituted by hydrogen ions, and the colloidal complex is partly destroyed, or reduced to its simple constituent oxides.

#### ALKALI INDICATORS

Native vegetation is a good indicator of the presence of alkali salts. HARRIS (16) states that there are at least 197 species native to California which are restricted to alkali soils. Some of these grow only when some particular salt is present, and others do well in the presence of a variety of alkali salts. In an arid region some plants may be found which indicate extremely large quantities of salts. Such plants are usually found alone. They indicate that so much alkali is present in the soil that agriculture without reclamation is impossible. According to HILGARD and others (22) crop plants may be grouped as follows:

- (a) Sensitive—maize, wheat, young lucerne, bur clovers, peaches, oranges, prunes, etc.
- (b) Resistant—oats, barley, sorghum, beets, etc.
- (c) Very resistant—saltbush (*Atriplex confertifolia*), couch grass, date palms, etc.

Native vegetation, coupled with chemical analysis of soils make an excellent combination in determining the degree of contamination of saline land.

#### TOXIC LIMITS OF SALINITY

The amount of salt a given plant will tolerate depends upon the conditions under which it is grown. Some such tests have been confined directly to field observations, some to laboratory tests, and others to water culture experiments.

The limits of plant tolerance for sodium carbonate, sodium chloride, and sodium sulphate, as reported by HILGARD (22) are 0.1 to 0.25, 0.3 to 0.5, and 0.5 to 1 per cent. respectively. The results of HIBBARD (21) show that much smaller amounts may be very injurious in some cases, and larger amounts may cause little effect in other cases. HARRIS (16) states that more than 0.5 per cent. soluble salts where predominantly chloride, nitrates, and carbonates, and 1 per cent. where sulphates predominate were unsuitable for crop production without reclamation.

BANCROFT (1) found the following concentrations of salts killed bean plants:



Magnesium chloride .....	2640 p.p.m.
Sodium carbonate .....	2710 p.p.m.
Sodium nitrate .....	3700 p.p.m.
Sodium chloride .....	3600 p.p.m.
Sodium sulphate .....	6510 p.p.m.
Sodium bicarbonate .....	12,300 p.p.m.

His results indicate that magnesium chloride is the most toxic, and sodium bicarbonate is the least toxic. BREAZEALE (2) also places magnesium chloride as the most toxic among the salts occurring in alkali soil. He holds that toxicity is the function of both molecules and ions and that the toxicity may be determined by the units of time the plants have come in contact with alkali in that form during their long period of adaptation. He also states that the limit of endurance of a plant to an alkali is based on the composition of the soil solution at the wilting point of the plant and not upon the percentage of dry soil. This would necessitate giving the wilting points. On the other hand, KEARNEY (24) believes that salines can be classified only on the basis of percentages of salts referred to the dry weight of the soil. BUFFUM (5), working with wheat in Wyoming, demonstrated that salts prevent absorption of water by seeds so that germination is markedly retarded. A low concentration of salts may aid germination.

#### TOLERANCE OF VARIOUS CROPS FOR SALTS

Obviously it is impossible to indicate the precise tolerance of any plant, except for a given condition. The capacity of any plant to endure alkali according to HIBBARD (18) varies with physical character of soil, kind, amount, and character of alkali, fertility of soil, moisture supply, climate, kind of crop, and other modifying factors.

BREAZEALE (3) maintains that salt tolerance in plants is largely a matter of adaptation and development of physiological differences, such as development of mechanisms for lowering the rate of transpiration, or loss of water through the leaves. DORE (6) found higher suberin content in roots of salt-tolerant plants.

From a practical standpoint a good criterion of the tolerance of a plant for alkali, according to NEIDIG and MAGNUSON (26), is the capacity of that plant to grow to maturity and produce good yields, rather than its response during the germination period only. This may be of value for the specific purpose of investigation, but may be misleading otherwise. KEARNEY (24) places a critical concentration of white alkali for staple field crops at 1 per cent. and for most plants 0.3 per cent. He classifies salinity into seven grades, depending upon the percentage of salts.

HIBBARD (20) classes grasses among the most salt resistant cultivated plants, although he recognizes blue grass (*Poa pratensis*) and a few others

that are rather sensitive. Bermuda grass (*Cynodon dactylon*) and Rhodes grass (*Chloris gayana*) are especially tolerant to sodium sulphate.

ZOBELL and STEWART (29) have shown that asparagus grew well in soils containing 10,000 p.p.m. of sodium sulphate. Barley and oats appeared to be most tolerant cereals, while sweet clover was more tolerant than alfalfa, beans, or peas. As a rule forage crops have proved to be better adapted to alkaline soil than most cultivated crops. Quality of fruit, vegetables, sugar, and grain crops are more definitely impaired by alkali salts.

HILGARD (22) states that presence of salts in quantities as small as 200 to 300 p.p.m. is generally harmful to legumes. He places the limit for growth at about 1650 p.p.m. of total salts; at about 300 p.p.m. of sodium carbonate; and at about 1390 p.p.m. of sodium sulphate.

KEARNEY (24) places the highest amount for successful growth at 4000 p.p.m. of white alkali. Alfalfa, however, is very sensitive to sodium carbonate at the seedling stage. Old stands of alfalfa may tolerate some 2000 to 7000 p.p.m. of total salts, according to various authors, the lower limit being for sandy soil of low buffer value. With sodium carbonate the toxic limits varied between 300 and 900 p.p.m., where the salt was mostly sodium chloride. The variation in salt concentration designated by various authors is from 200 p.p.m. on sandy soil to 7100 p.p.m. on loam soil well supplied with moisture. Sweet clover seems to succeed better than alfalfa on alkali soils that are water logged or have shallow water table.

In explanation the literature on the subject of salt tolerance reveals that the studies may be grouped into two categories: (a) the amount of various salts added; and (b) the amount of salt recoverable in the water extract of the soil. Later studies indicate that only part of the added salts is recovered in water extract; hence the ratio of recovered to the total salts may not be the same as was revealed from salt additions.

HEADLY, CURTIS, and SCOFIELD (19) found that sodium carbonate added to the soil and allowed to remain several weeks is only partly recovered with the water extract of the soil. The limit of tolerance of crops to salts can best be determined by the amount of salt recovered from the soil rather than by the quantity added. The proportion of recoverable salts which killed half of the wheat seedlings was 0.04 per cent. for carbonates, 0.16 per cent. for chloride, and 0.35 per cent. for sulphate.

HARRIS and PITTMAN (18) have pointed out the discrepancy between the amount of added alkali salts and that recoverable in the water extract after such additions. They also found greater injury to plants grown in sand than to those grown in loam soil after each received the same amount of sodium carbonate.

#### TOLERANCE OF BACTERIA FOR SALTS

Effect of various salts upon the activity of ammonifying and nitrifying bacteria and other bacterial processes in the soil has been extensively investi-

gated. LIPMAN (25) reported sodium chloride toxic to ammonification by *B. subtilis*, and that the toxicity was reduced by the addition of magnesium or potassium chloride. Sodium carbonate was found stimulative in concentrations as high as 1 per cent., while 0.2 per cent. sodium sulphate reduced ammonification nearly one-half, and 0.2 per cent. sodium chloride reduced ammonification about two-thirds. BROWN and associates (4) found 0.06 per cent. calcium carbonate stimulating to ammonifiers, while 0.2 per cent. sodium carbonate, 0.1 per cent. sodium bicarbonate, and 0.5 per cent. sodium sulphate inhibited ammonification. GREAVES (15) found relative toxicity of various salts to nitrification decreased as the amount of moisture increased. GIBBS and associates (10) found toxicity of sodium carbonate to bacterial activities varied and was more tolerant toward the later period of growth. Change in chemical composition of protoplasm or in osmotic pressures produced may prevent bacteria from performing their normal functions, according to GREAVES, who found azofiers more resistant to alkali or neutral salts than ammonifiers, nitrifiers, and most higher plants. The common soil alkali, before retarding nitrogen fixation, then, would have to be present in quantities sufficient to greatly retard ammonification, nitrification, and plant growth.

### Materials and methods

Experiments were undertaken to study temperature and other factors affecting the salt tolerance of salt grass (*Distichlis spicata*), alfalfa (*Medicago sativa*), sweet clover (*Melilotus* sp.), strawberry clover (*Trifolium fragiferum*), and a legume from Montana (*Astragalus rubyii*). Water culture, soil culture, and field plots were included.

Preliminary experiments were conducted with sea water, diluted to different concentrations with duplicate series of each concentration provided. The Pacific Ocean water after dilution was enriched by addition of Shive's culture solution of a strength equivalent to an osmotic concentration of one-tenth atmosphere. The reaction was adjusted and held at pH 5.7 to 6. Duplicate jars of "complete" nutrient solution equivalent to one atmosphere concentration were included as checks. In addition sodium chloride and sodium carbonate plus dilute culture solutions were employed. Two-gallon jars were used in all cases. Seeds were germinated in quartz sand and transplanted approximately when the fourth leaf appeared. One set of cultures was placed in a cold plant-house with a temperature of 55° F. and another was kept in a warm greenhouse at a temperature of 70°.

A constant temperature germinator built for Neubauer tests was made available for germination experiments under controlled conditions of temperature. Strawberry clover was germinated, and after eighteen days the percentage of germination was determined. Experiments were conducted

at 55, 70, and 90° F. The latter temperature proved so unfavorable that a second trial was made at a temperature of 85° F.

In addition a constant-flow experiment was conducted using sea water at various dilutions. The apparatus was adjusted to deliver a flow of some 2 liters per day.

Supplies of surface soil at the Vale Alkali Experiment Field were collected in June, 1936, from plots A, B, and N, and adjacent native hard alkali land previously described by MOUNSTON and POWERS (23).

Another lot sample of sodium Yakima loam soil was obtained from near Klamath Falls. These soils were brought to the greenhouse, each lot mixed thoroughly and placed in duplicate series of one-gallon jars. Various chemical treatments were given to the soil to provide a series of pH values ranging from native alkali soil to neutrality. After planting, a favorable moisture content was maintained.

Chemical analyses of soil profile layers from the field tracts were made as to base exchange capacity, replaceable bases, water soluble salts, organic matter, available nutrients, and pH. Base exchange was determined by the barium chloride-ammonium chloride method, organic matter by Alexander's modifications of the Nether method, and pH with the hydrogen electrode. Certain biological activities of these alkali soils were studied. Detailed results, however, are to be reported separately.

Some moisture equivalent determinations were made using the centrifuge method.

The average salt content of sea water used for water culture experiments was 3.06 per cent. or 30,600 p.p.m. The dilutions used and dry weights of salt grass produced in the cold house and also in the warm house are shown in table I.

TABLE I  
TOLERANCE OF SALT GRASS FOR SEA WATER

CONCENTRATION OF SEA WATER	WEIGHT OF DRY MATTER, AVERAGE OF 2 CULTURES (FIRST CUTTING)		WEIGHT OF DRY MATTER, AVERAGE OF 2 CULTURES (SECOND CUTTING)		TOTAL WEIGHT	
	COLD HOUSE	WARM HOUSE	COLD HOUSE	WARM HOUSE	COLD HOUSE	WARM HOUSE
	gm.	gm.	gm.	gm.	gm.	gm.
Complete nutrient solution .....	47.0	21.2	49.0	25.3	96.0	46.5
306 p.p.m. plus dilute nutrient solution .....	14.4	8.3	21.2	12.5	35.6	24.8
612 p.p.m. plus dilute nutrient solution .....	14.3	7.7	20.0	11.1	34.3	18.8
1834 p.p.m. plus dilute nutrient solution .....	12.7	7.7	13.1	9.5	25.8	17.2
2448 p.p.m. plus dilute nutrient solution .....	11.3	3.4	10.5	3.6	21.8	7.0

## Experimentation

### WATER CULTURE EXPERIMENTS

It is very interesting to note that in the cold house the total weight of dry matter obtained in the highest concentration of sea water is more than three times as much as that in the warm house. A significant difference in weight of dry matter of salt grass is shown in the various concentrations of sea water used (table I). The critical concentration of sea water for the conditions appears to be approximately 2448 p.p.m.

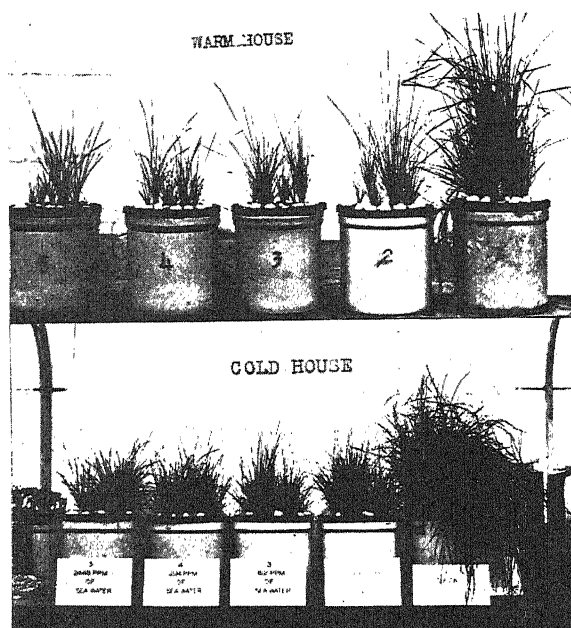


FIG. 1. Above, salt grass growing in various concentrations of sea water in warm house (about 75° F.). 1, check; 2, 306 p.p.m. of sea water; 3, 612 p.p.m. of sea water; 4, 1224 p.p.m. of sea water; 5, 2448 p.p.m. of sea water. Below, salt grass growing in the same concentrations of sea water in the cold house (about 55° F.).

This type of experiment was repeated with alfalfa using different concentrations of sodium chloride, sodium sulphate, and sodium carbonate as shown in tables II and III. The critical concentrations indicated by these tests are summarized in table IV. Combinations of different ratios of sodium carbonate to sodium sulphate indicate higher tolerance in combinations. These concentrations are lower than reported by HARRIS (20) for soil cultures. They are also lower than the concentrations of brackish water at high tide found bathing areas of salt grass growing on lowlands near the Pacific Coast in Oregon.

TABLE II  
TOLERANCE OF ALFALFA FOR SEA WATER

CONCENTRATION OF SEA WATER	WEIGHT OF DRY MATTER, AV. OF 2 CULTURES (FIRST CUTTING)	WEIGHT OF DRY MATTER, AV. OF 2 CULTURES (SEC- OND CUTTING)	TOTAL WEIGHT
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Complete nutrient solu- tion .....	13.4	18.3	31.7
350 p.p.m. plus dilute nutrient solution .....	13.1	16.1	29.2
700 p.p.m. plus dilute nutrient solution .....	13.8	11.3	25.1
1400 p.p.m. plus dilute nutrient solution .....	7.2	5.5	12.7

TABLE III  
TOLERANCE OF ALFALFA FOR SODIUM CHLORIDE

CONCENTRATION OF SODIUM CHLORIDE SOLUTION	WEIGHT OF DRY MATTER, AV. OF 2 CULTURES (FIRST CUTTING)		WEIGHT OF DRY MATTER, AV. OF 2 CULTURES (SEC- OND CUTTING)		TOTAL WEIGHT	
	COLD HOUSE	WARM HOUSE	COLD HOUSE	WARM HOUSE	COLD HOUSE	WARM HOUSE
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Complete nutrient so- lution .....	15.0	13.0	15.8	13.6	30.8	26.6
350 p.p.m. plus dilute nutrient solution .....	13.5	11.9	14.7	11.1	28.2	23.0
700 p.p.m. plus dilute nutrient solution .....	11.8	9.4	13.4	10.6	25.2	20.0
1400 p.p.m. plus dilute nutrient solution .....	4.0	2.6	8.9	5.7	12.9	8.3

TABLE IV  
CRITICAL SALT CONCENTRATIONS FOR CERTAIN PLANTS

PLANTS	CONCENTRATIONS			
	SEA WATER	SODIUM CHLORIDE	SODIUM SULPHATE	SODIUM CARBONATE
	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>
Salt Grass .....	10,000	1400	2800	700
Alfalfa .....	2800	1000	1400	350
Strawberry Clover ...	5600	.....	.....	.....

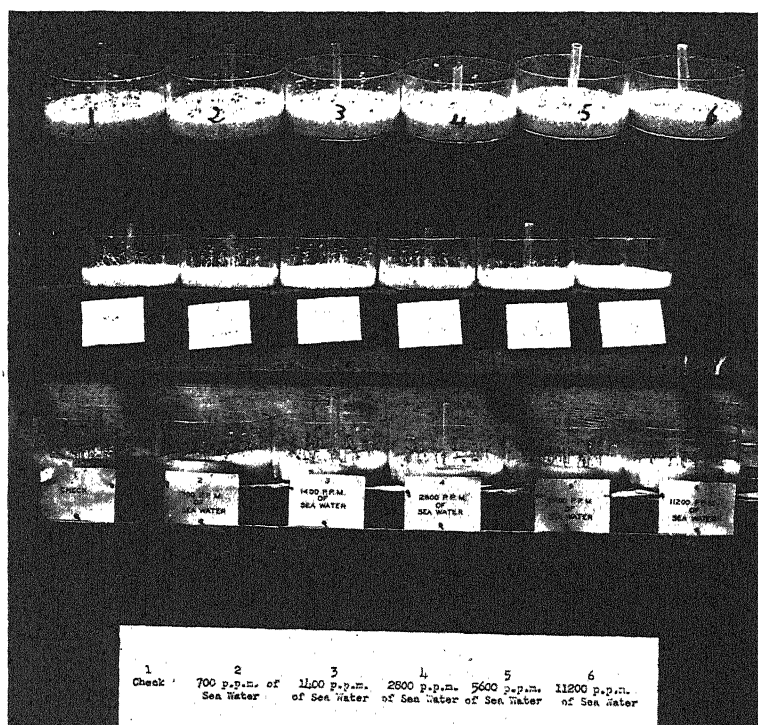


FIG. 2. Top row, strawberry clover grown at different concentrations of sea water increasing from left to right as indicated, at about 85° F. Middle row, same series of concentrations, germinated at 70° F. Bottom row, same series of concentrations, germinated at 55° F.

TABLE V

TOLERANCE OF STRAWBERRY CLOVER FOR SEA WATER AT CONSTANT CONCENTRATION

CONCENTRATION OF SEA WATER	WEIGHT OF DRY MATTER, AV. OF 3 CULTURES* (FIRST CUTTING)	WEIGHT OF DRY MATTER, AV. OF 3 CULTURES (SECOND CUTTING)	TOTAL WEIGHT
Complete nutrient solution .....	gm.	gm.	gm.
700 p.p.m. plus dilute nutrient solution	4.0	6.50	10.50
1400 " " " " " "	2.67	2.95	5.62
2800 " " " " " "	3.00	3.50	6.50
5600 " " " " " "	1.00	2.10	3.10
11200 " " " " " "	0.75	1.50	2.25
	0.50	0.50	1.00

\* Only five seedlings were planted in each jar.

Strawberry clover is one of the more promising alkali-resistant legumes. It has been grown in recent field trials with fair success on Oregon soils with a pH up to 9 to 9.8 and also on acid soils with a pH value as acid as pH 4.2.<sup>3</sup> The tolerance of this plant for sea water in flowing solution cultures regulated to provide constant concentrations is shown in table V.

The critical concentration of salts which this plant may tolerate, under the conditions of the experiment, is indicated to be approximately 5600 p.p.m.

#### SAND CULTURE EXPERIMENTS

From the better results secured in the cold house as compared to the warm house with a difference in mean temperature of approximately 15° F., it seemed desirable to further investigate this factor. A series of germination tests were, therefore, conducted using sand cultures in a germinator with temperature control. Strawberry clover and alfalfa seeds were used in these experiments. Lots of 100 seeds were germinated in clean quartz sand within large petri dishes for a period of 17 days. Water was added daily to maintain an even concentration and keep the cultures moist for germination. At the end of the period seedlings were counted and germination percentages calculated. The effect of temperature upon germination of seeds at various concentrations of sea water is shown in table VI. This

TABLE VI

EFFECT OF TEMPERATURE ON GERMINATION OF SEEDS AT VARIOUS CONCENTRATIONS OF SEA WATER

CONCENTRATION OF SEA WATER	STRAWBERRY CLOVER SEEDS GERMINATED			ALFALFA SEEDS GERMINATED		
	55° F.	70° F.	90° F.	55° F.	70° F.	90° F.
No sea water .....	%	%	%	%	%	%
700 p.p.m. of sea water .....	89.5	85.0	20.0	94.0	90.0	18.3
1400 p.p.m. " " " .....	95.0	87.0	5.0	93.0	82.2	3.0
2800 p.p.m. " " " .....	98.0	67.0	0.0	90.0	69.8	0.0
5600 p.p.m. " " " .....	85.3	79.0	0.0	90.0	38.5	0.0
11200 p.p.m. " " " .....	63.5	40.0	0.0	76.0	15.3	0.0
	47.7	0.0	0.0	38.0	0.0	0.0

effect is well illustrated in figure 2 where the relationship is shown more definitely than in the table by its effect on growth.

The effect of temperature is very striking. There is a definite decrease in the percentage of germination with increase in temperature or salt concentration. In high salt concentrations decrease occurs very rapidly at

<sup>3</sup> Tests of the tolerance of strawberry clover were made in cooperation with the Division of Forage Crops and Diseases, Bureau of Plant Industry, U. S. Department of Agriculture.





FIG. 3. Growth of alfalfa on alkali land, check plot at left; reclaimed with sulphur at right, at Vale, Oregon, August, 1937.

high temperatures, but relatively slowly at low temperatures. At 90° F. there was practically no germination of seeds in the various treated cultures, while at 55° F. there was a high percentage of germination, except at the highest concentration. At the highest concentration there was no germination except with the lowest temperature which resulted in a germination of 47.7 per cent. for strawberry clover seeds, and of 38 per cent. for alfalfa. The two lower concentrations of sea water were stimulative to strawberry clover, but slightly retarded germination of alfalfa.

In comparing the two legumes, it will be noted that strawberry clover seeds germinate more satisfactorily than alfalfa in higher concentrations of sea water. In the presence of 2800 p.p.m. of total salts at ordinary growth temperature, strawberry clover germination in these tests was 78 per cent., as compared to 38.5 per cent. for alfalfa seeds. The strawberry clover is definitely more tolerant than the other seedlings tested and will germinate fairly well in the presence of as high as 5600 p.p.m. of total salts. Under cool and moist conditions, it may germinate in the presence of much higher concentrations.

#### SOIL CULTURE EXPERIMENTS

Series of soil culture experiments were initiated in the spring of 1936 using strawberry clover as indicator plants. Two soils were included in these investigations. One of these was an alkali soil brought from Vale Experiment Field and the other, a saline soil, was taken from near Klamath Falls. Both were loams. The initial reactions and chemical characteristics of these soils are presented in tables IX, X, and XI. Treatments were applied to give a reaction range or pH from nearly neutral to strongly alka-

line as shown in tables VII, VIII, and IX. The final soil reactions and results are also given in these tables.

TABLE VII

TOLERANCE OF STRAWBERRY CLOVER IN ALKALINE YAKIMA LOAM POT CULTURE

TREATMENT	WEIGHT OF DRY MATTER, AV. OF 2 CULTURES (FIRST CUTTING)	WEIGHT OF DRY MATTER, AV. OF 2 CULTURES (SECOND CUTTING)	TOTAL DRY WEIGHT	FINAL SOIL REACTION
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>pH</i>
No treatment .....	5.50	6.00	11.50	8.63
Sulphur, 1 T.* .....	6.00	7.00	13.00	7.61
Sulphur, 1 T.; manure, 20 T. ....	6.75	8.50	15.25	7.32
NaCl, 1000 p.p.m. ....	0.50	2.50	3.00	9.35
NaCl, 2000 p.p.m. ....	0.00	0.70	0.70	9.56
Na <sub>2</sub> CO <sub>3</sub> , 500 p.p.m. ....	0.00	1.20	1.20	9.48
Na <sub>2</sub> CO <sub>3</sub> , 1000 p.p.m. ....	0.00	0.40	0.40	9.78

\* Tons per 2,000,000 lb. of soil.

Obviously the application of sulphur or sulphur with manure resulted in a greater yield of dry matter. This increase in dry matter was higher in the second cutting of the crop than in the first. The effect of sulphur with manure was more pronounced than that of sulphur alone.

The increase of dry matter due to the application of sulphur and sulphur with manure in the Yakima loam soil cultures was moderate while the effect was very marked in the Vale soil where it increased yields approximately threefold. The reactions induced would tend to reduce alkalinity but

TABLE VIII

TOLERANCE OF STRAWBERRY CLOVER IN LOAM SOIL FROM PLANT N, VALE  
EXPERIMENT FIELD

TREATMENT	WEIGHT OF DRY MATTER, AV. OF 2 CULTURES (FIRST CUTTING)	WEIGHT OF DRY MATTER, AV. OF 2 CULTURES (SECOND CUTTING)	TOTAL DRY WEIGHT	FINAL SOIL REACTION
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>pH</i>
No treatment .....	2.60	4.28	6.88	8.50
Sulphur, 1 T.* .....	5.10	6.75	11.85	7.39
Sulphur, 1 T.; manure, 20 T. ....	7.00	8.50	15.50	7.10
NaCl, 1000 p.p.m. ....	4.30	5.00	9.30	8.73
Na <sub>2</sub> CO <sub>3</sub> , 500 p.p.m. ....	3.10	2.50	5.60	8.82

\* Tons per 2,000,000 lb. of soil.

TABLE IX

RESPONSE OF VARIOUS LEGUMES IN ALKALINE SOILS WITH OR WITHOUT TREATMENTS

SOIL	TREATMENT	DRY WEIGHT OF ALFALFA, AV. OF 2 CULTURES	DRY WEIGHT OF STRAW- BERRY CLOVER, AV. OF 2 CULTURES	DRY WEIGHT OF SWEET CLOVER, AV. OF 2 CULTURES	DRY WEIGHT OF <i>Astra- galus rubyii</i> , AV. OF 2 CULTURES
		gm.	gm.	gm.	gm.
Virgin alkaline soil, Vale Experi- ment Field	No treatment .....	0.25	2.25	1.40	0.0
	Sand (200%) .....	3.35	3.98	3.70	0.0
	Gypsum (12 T.)* .....	5.20	5.05	5.50	0.0
	Sulphur ( 2 T.).....	5.50	5.87	6.95	0.0
	Sulphur ( 2 T.) }	7.90	8.86	12.90	4.1
	Manure (20 T.) }				
Plat A Vale Experi- ment Field	No treatment .....	6.00	6.70	6.35	0.0
	Sand (200%) .....	6.30	7.15	6.20	0.0
	Gypsum (12 T.).....	6.40	8.15	6.90	0.0
	Sulphur ( 2 T.).....	6.70	8.10	6.40	1.25
	Sulphur ( 2 T.) }	6.75	8.90	7.70	5.2
	Manure (20 T.) }				
Plat B Vale Experi- ment Field	No treatment .....	2.90	3.20	2.10	0.0
	Sand (200%) .....	3.30	3.67	3.15	0.0
	Gypsum (12 T.).....	4.95	5.00	3.40	0.0
	Sulphur ( 2 T.).....	5.00	5.17	3.70	0.0
	Sulphur ( 2 T.) }	5.50	5.90	4.20	4.6
	Manure (20 T.) }				

\* Tons per 2,000,000 lb. of soil.

might increase total soluble salts. More growth was secured on untreated Yakima loam than on the check jars of more alkaline Vale loam, which was in a poor physical condition.

Sodium chloride and sodium carbonate treatments were detrimental to the growth of plants in both soils, although 1000 p.p.m. of sodium chloride did not decrease the total yield of dry matter on Vale soils.

From the data, it may be concluded that strawberry clover will make a better growth on alkaline, or saline soils, provided the soil is well supplied with sulphur and manure accompanied by leaching. Sulphur oxidizes and brings calcium into solution to replace exchangeable sodium. One effect of manure is to supply microorganisms for biological oxidation of the sulphur.

#### CHEMICAL ANALYSES OF SOILS

The soils that were used in the greenhouse were sampled in the field by horizons, 0 to 5, 5 to 20, and 20 to 40 inches depth. Determinations of replaceable bases, soluble salts, reaction, total nitrogen, and organic matter, as

TABLE X  
 REPLACEABLE BASES IN VALE ALKALI EXPERIMENT PLATS. SAMPLED IN FALL, 1936

PLAT	TREATMENT	DEPTH	BASE EX- CHANGE CAPACITY PER 100 GM.	EXCHANGE CA - MG PER 100 GM.	EXCHANGE NA PER 100 GM.	EXCHANGE K PER 100 GM.	CA AS PART OF EXCHANGE CAPACITY	NA AS PART OF EXCHANGE CAPACITY
Virgin alkali soil		<i>inches</i>	<i>M.E.</i>	<i>M.E.</i>	<i>M.E.</i>	<i>M.E.</i>	%	%
		0 - 5	24.25	2.48	20.26	1.51	10.22	83.13
		5 - 20	28.60	0.65	26.76	1.19	2.27	93.56
Plat A	Manure (10 T.) 6 yrs. (60 T.) Sulphur (1.5 T.)	20 - 40	22.61	2.05	19.50	0.96	9.06	86.68
		0 - 5	20.75	15.95	4.10	0.70	76.87	19.76
		5 - 20	22.26	16.84	4.72	0.70	75.65	21.20
Plat B	Untreated	20 - 40	24.17	20.78	3.02	0.39	85.90	12.49
		0 - 5	27.49	4.75	21.09	1.65	17.28	76.72
		5 - 20	32.34	5.93	24.15	2.26	18.34	77.77
Plat N*	Sulphur (500 lb.)	20 - 40	33.34	12.84	18.19	1.31	39.69	56.25
		0 - 5	12.94	9.78	2.73	0.43	75.50	21.10
		5 - 20	18.33	13.74	3.14	1.45	74.90	17.10
Yakima loam† from Klamath Falls		20 - 40	24.02	21.00	2.90	0.16	87.50	12.50
		0 - 6	19.94	5.80	12.51	1.63	29.10	62.74
		6 - 18	17.79	8.60	8.05	1.14	48.31	45.23
		18 - 28	17.25	8.33	7.93	0.94	48.28	46.51

\* Sampled in April, 1936.

† Sampled in June, 1936.

TABLE XI

WATER-SOLUBLE SALTS IN VALE ALKALI EXPERIMENT PLATS. SAMPLED FALL, 1936

PLAT	DEPTH	TOTAL SOLUBLE SALTS	CAR- BONATE (CO <sub>3</sub> )	BICAR- BONATE (H CO <sub>3</sub> )	SUL- PHATE (SO <sub>4</sub> )	CHLOR- IDE (Cl)	SODIUM (Na)
	<i>inches</i>	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>	<i>p.p.m.</i>
Virgin	0 - 5	3150	1019	.....	78	108	1200
alkali	5 - 20	4700	811	.....	1197	427	1509
soil	20 - 40	3480	811	.....	607	230	1345
	0 - 5	560	0	82	78	55	396
	5 - 20	960	0	82	66	56	387
Plat A	20 - 40	880	0	41	102	47	453
	0 - 5	2950	811	1237	210	95	1057
	5 - 20	3500	761	1016	535	273	1369
Plat B	20 - 40	2020	406	412	197	148	2121
	0 - 5	1310	0	330	82	76	969
	5 - 20	930	0	491	87	52	1465
Plat N*	20 - 40	1320	0	742	107	57	280
Yakima loam†	0 - 6	4010	0	2474	119	109	1294
from	6 - 18	1980	Trace	1640	99	58	948
Klamath Falls	18 - 28	1710	Trace	1237	99	57	671

\* Sampled in April, 1936.

† Sampled in June, 1936.

well as moisture equivalent were made. In addition, the relationship of exchangeable sodium and exchangeable calcium were calculated and reported. The object of this investigation was to determine the extent of alkalinity or progress of reclamation and also to correlate the results with the general growth of plants on such soil.

REPLACEABLE BASES.—The amount and kind of adsorbed bases is one of the important properties indicating the extent of alkalinity or progress of reclamation. Table X shows the content of replaceable bases and the proportion of univalent cations (Na) and divalent cations (Ca-Mg) to the total exchange in all soil profiles. Plat A which was treated with sulphur and manure is now largely saturated with calcium, while plat B is still mostly saturated with sodium. The proportion of divalent cations in the 40-inch profile is much higher in plat A than in plat B. The replaceable sodium shows a reverse relationship indicating little modification in plat B from virgin soil. The exchange of calcium for sodium is most nearly complete in plat A which receives most sulphur and manure. A similar relationship exists in plat N indicating that alkaline soils could be effectively reclaimed. The treated plats show good exchange capacities, although

lower than those of virgin alkali samples. The high content of replaceable sodium in virgin soils from such plats will explain the high alkalinity of the soil. The Yakima loam soil from Klamath Falls contains replaceable sodium as well as salts to account for the unproductivity of the soil.

**WATER SOLUBLE SALTS.**—The soluble salts in the various horizons of different profiles are shown in table XI. There is marked difference between the check plat and the treated plat. The check plat contains more than five times as much water-soluble salts in the surface layer as that in plat A. There is slight difference in the total salt content between the check plat and virgin soil. Plat N, although the salt content is much lower as compared with the check plat, contains sufficient sodium salts to be a factor in the abnormality of the soil. In both treated plats the alkaline carbonate has disappeared throughout the whole profile studied. There is little change in alkaline carbonate in the check plat as compared to the virgin soil. Sulphates and chlorides are markedly diminished in the treated plats. In most every case both sulphates and chlorides are concentrated in the 5- to 20-inch layer of the soil profile. The relative amount of soluble sodium removed from the check plat has been very slight. Only with chemical treatment is there a marked elimination of soluble sodium. The Yakima loam soil shows a higher salt content than the treated land from Vale.

The salt content of the surface layer is even higher than that of the virgin alkaline soil. It appears that the Yakima loam soil is of a saline type as compared with the alkaline soils from Vale.

**AVAILABLE NUTRIENTS.**—The available nutrients in saline or alkaline soils are almost invariably low and the reaction is too alkaline for best growth of crop plants in many cases. The low content of nitrogen is the natural result of sparse vegetative cover which the soil supports. The effect of treatment on available nutrients is well illustrated in table XII.

It will be noticed that the reaction of the soil of plat A is approaching neutrality. The reaction of the two treated plats in general is well within the growth range for alfalfa. There is a relative increase in total nitrogen in treated plats as compared with that of the checks as reported earlier by POWERS (28). The increase in total nitrogen is due to a corresponding increase in organic matter. The soluble phosphorus is higher in the virgin soils than the other plants. This is perhaps due to the phosphorus being in the form of sodium phosphate, and also the plats may have lost some phosphates. The effect of treatment is further indicated by the amount of soluble calcium. In all layers the treated plats contain approximately twice as much soluble calcium as that of the checks. This shows that there is a tendency for the added material to bring calcium into solution.

The effect of reclamation is well demonstrated in figure 3. This relationship is more apparent in table XIII where the yields of hay per acre

TABLE XII  
AVAILABLE NUTRIENTS IN VALE ALKALI EXPERIMENT PLATS. SAMPLED FALL, 1936

PLAT	DEPTH	REACTION	TOTAL NITROGEN	ORGANIC MATTER	WATER SOLUBLE PHOSPHORUS AS $PO_4$	WATER SOLUBLE CALCIUM	MOISTURE EQUIVALENT
	inches	<i>pH</i>	%	%	<i>p.p.m.</i>	<i>p.p.m.</i>	%
Virgin ..... alkali soils	0 - 5	9.87	0.054	1.31	13.9	76	29.3
	5 - 20	9.89	0.028	1.17	32.3	82	27.3
	20 - 40	9.80	0.024	1.02	27.8	82	30.1
Plat A	0 - 5	7.60	0.093	2.39	6.1	122	34.1
	5 - 20	8.50	0.056	1.32	5.0	90	37.1
	20 - 40	8.77	0.028	0.91	16.1	111	35.4
Plat B	0 - 5	9.40	0.046	1.08	18.9	56	26.9
	5 - 20	9.92	0.044	1.08	1.1	68	33.7
	20 - 40	9.32	0.028	1.01	3.0	62	36.1
Plat N*	0 - 5	8.75	0.123	1.34	10.4	125	.....
	5 - 20	8.15	0.079	1.46	3.0	105	.....
	20 - 40	8.18	0.082	1.25	8.9	119	.....
Yakima loam† from Klamath Falls	0 - 6	9.45	0.107	2.48	8.9	121	.....
	6 - 18	9.10	0.076	1.70	18.9	68	.....
	18 - 23	9.05	0.044	0.82	12.3	64	.....

\* Sampled in April, 1936.

† Sampled in June, 1936.

TABLE XIII

YIELDS OF ALFALFA HAY SECURED ON VALE ALKALI EXPERIMENT PLATS FROM  
1931 TO 1936

PLAT	TREATMENT	POUNDS PER ACRE					
		YEAR					
		1931	1932	1933	1934	1935	1936
		lb.	lb.	lb.	lb.	lb.	lb.
A	Manure (10 T.) 6 yrs. (60 T.) Sulphur (1.5 T.)	3720	3180	6480	7112	4902	4860
B	Untreated	0	220	680	610	780	1256
C	Manure (8 T.) 5 yrs. (40 T.) Sulphur (1500 lb.)	2020	4600	3060	3220	3790	5292

obtained on plats A and B for a period of years is presented. It will be noted that in each the yield obtained in plat A is much higher than that obtained from plat B. Although there is some seasonal variation, in part due to water shortage and injury to the stand by freezing, still the treated plat has produced rather good yields for the locality. General indications are very promising as to the ultimate success of reclamation. In these plats, alfalfa tends to become chlorotic when the reaction value of pH 8.6 or higher is obtained. Sulphur and manure, when used together, have been very effective in the reclamation of these alkaline soils.

Where strawberry clover seedlings were chlorotic the soil tested pH 9.2, and where color of seedlings was normal it tested 9 early in the season. In September chlorotic plants were found on soil that had a pH of 10. At this time plants of good color were found to be maturing seed on nearby soil having a pH value of 9.71. These tests were with samples obtained near Klamath at 4100 feet elevation.

Samples taken from soils near Vale at 2200 feet elevation June 10, where strawberry clover seedlings had good color, showed a pH value of 8.8; and where seedlings were chlorotic, the soil pH was 9.3. In September strawberry clover plants appeared thrifty at a pH value of 8.83, while a soil on which chlorotic plants were growing had a pH value of 9.15. A seed yield of 300 pounds per acre was obtained near Vale.

Near Baker, at 3400 feet elevation, strawberry clover in September, 1937, appeared thrifty with soil pH of 9.45 and chlorotic with soil pH of 9.99.

Prosser Station, Washington, reports (mimeo 17th annual field data July 9, 1937): "the average concentration of salts in soils that strawberry clover plants will tolerate is 0.4 per cent."



### MICROBIAL STUDIES<sup>4</sup>

Studies of the effect of alkali salts on the activities of desirable soil microorganisms will be treated only briefly. A poor physical condition of alkali soils may check the activity of such organisms, and the concentration of salts may result in plasmolysis. Data from table XII show an improvement in moisture equivalent, indicating improved structure, water capacity, and perhaps carbon dioxide evolution resulting from the partial reclamation of certain plats, such as plat A.

Various materials were applied to these alkali soils and observations made as to the carbon dioxide evolved as well as the change in number of bacteria and molds. In general, the various treatments were effective in increasing the number of microorganisms.

There was a significant difference in the sulphur-oxidizing power of the soil. At the close of the experiment 100 per cent. of the sulphur added in the reclaimed soil was oxidized while only 20 to 30 per cent. of the sulphur was oxidized in the virgin soil. Sulphur oxidation in both soils was most effective in the first 115-day period as compared with the second interval of 100 days.

This was not true so far as *Azotobacter* is concerned. No development of these organisms occurred during the first period while in the second period moderately heavy development occurred. In general, the various treatments were effective in increasing the number of microorganisms. Similar results were obtained in the reclaimed land with the exception that in most cases moderate development of *Azotobacter* occurred in the second period as compared to the heavy development in the first period. The treatment increased the amount of water-soluble phosphorus, especially where sulphur was applied.

### Discussion

From results obtained with salt-tolerance studies, temperature and other factors have been shown to be of vital importance. Results should be of practical application in selection of soils or providing conditions favorable for plant growth.

### NATURE OF INJURY

Soluble salts may cause injury to plants by preventing them from absorbing moisture; by direct corrosive action; by toxicity due to excess of salt or its ions, or the products of hydrolysis; by inhibiting biological activities; by their effect upon germination; or by producing an abnormal physical condition in the soil. Seeds may germinate in saline soil and the young seedlings may be killed by the high and changing concentration of the soil

<sup>4</sup> Dr. W. B. BOLLEN, of the Oregon State College, Department of Bacteriology, supervised the microbial studies reported herein.

solution. In general older plants are more resistant than young seedlings. Plants growing on alkaline soil have characteristics similar to those found under desert conditions. They lack the bright green appearance of vigorous, healthy plants.

Under the conditions of the experiment the concentration of sodium carbonate which the salt grass may tolerate is 700 p.p.m. It may be possible that under field conditions the mature plants would do well in higher concentrations than results here would indicate. The limit of tolerance of salt grass has been found to be 1400 p.p.m. for sodium carbonate and 2800 p.p.m. for sodium sulphate.

Alfalfa, although sensitive to salinity at the seedling stage, made a fairly good growth in various concentrations of salt with indications of better growth under cooler conditions.

Results of sand culture tests clearly indicate (a) that strawberry clover is superior under alkaline or saline conditions to alfalfa, and (b) that temperature plays an important part in the germination and growth of plants in saline soils. Further evidences are found from concentration experiments using sea water, as clover, on the soil used, is found to tolerate a concentration as high as 5600 p.p.m. of total salts. The results are affected by temperature for the germination is not so good with more heat. It seems sufficient here to present conclusive evidence that differences in salt tolerance occur with changes in temperature. Water-culture experiments with salt grass and alfalfa present further confirmatory evidence that temperature is an important factor in the germination and growth of plants under saline conditions. The following suggestions are offered to the effect that temperature may be due to the action of one or the combined action of three or more factors. In the first place, it may be attributed to the "heat" factor only, since there is a reduction in the seed germination even in the check. This factor may be active only at a temperature of 90° F. because at 73° F., which is the normal growth temperature, no detrimental effect is noted due to the heat. Since the reduction of approximately 80 per cent. germination occurred in the checks at 90° F. it seems safe to state that heat is a factor.

In the second place, the relationship of temperature to germination may be due to the function of both the molecules and the ions. Previous evidence by BREAZEALE (2) supports the above statement. It is well understood that rise in temperature will increase the activity of ions due to the high degree of dissociation of the salt. The sea water is largely composed of easily ionizable salts and an increase of 20° F. should increase the number of ions present. This greater concentration of ions may cause greater toxicity; hence the complete retardation of germination.

Finally, the prevention of germination at 90° F. may be due to the rate

of mobility or diffusion of the salt. An increase in temperature results in an increase in the rate of diffusion which may ultimately cause the complete inhibition of germination. Possibly the increased activity of ions may be the only logical explanation of the temperature effect upon the germination of seed. Fertilizer burning in hot weather is closely related to salt tolerance. Tolerance will vary with age and kind of crop.

The results of chemical analyses and those of salt tolerance studies are not in full agreement. Improved chemical conditions in the soil will increase yields in the field. It was not fully apparent under greenhouse conditions, but this may be due to the following factors:

(a) The physical abnormality of the soil. When a comparative trial was made on the same soil diluted with sand the crop made a better growth.

(b) The lack of desirable microorganisms or their activities in the soil. This is indicated by the best growth of crops in pots treated with sulphur and manure, wherein the manure may have harbored microorganisms that speed up desirable microbiological activities.

(c) Replaceable sodium. Even in the better plats replaceable sodium is still present in moderate amounts.

(d) Under greenhouse conditions soil, light, relative humidity, and other factors are modified.

The virgin alkali plat soil was found to harbor an active and versatile microbial population. There was a significant difference, however, in the oxidizing power of the soils. The sulphur-oxidizing power of reclaimed soil is much greater than in virgin land. Salts applied to the Willamette silty clay loams in western Oregon had a depressing effect upon microbial activity in the soil. All of the salts used showed an inhibiting effect upon numbers and activities of microorganisms, sodium chlorate being the most effective.

### Summary

1. Temperature is a dominant factor in the germination and growth of plants under saline or alkaline conditions. Temperature relations should be considered before recommending certain crops for saline soils.

2. Strawberry clover was found the most promising resistant legume for salinity, followed by sweet clover, then alfalfa. *Astragalus rubyii* failed to grow well under different treatments, with the exception of the soil receiving sulphur and manure.

3. Chemical analyses show a marked improvement of treated plats over those of checks by restoration of calcium for sodium in the exchange complex, improved reaction, decreased salt concentration, higher level of organic matter and nitrogen, a tendency to increase base exchange capacity, and useful water capacity or moisture equivalent. The crop yields obtained under field conditions substantiate the chemical results.

4. The sulphur oxidizing power of reclaimed soil was found to be much greater than that of the virgin alkali soil. Various treatments increased nitrate nitrogen.

OREGON AGRICULTURAL EXPERIMENT STATION  
CORVALLIS, OREGON

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# TRANSLOCATION IN PLANTS

A. S. CRAFTS

## Introduction

Elimination of certain untenable theories has narrowed recent research on translocation to a search for the mechanism of rapid longitudinal movement of foods through phloem. (For comprehensive bibliographies see 22, 48, and 51.) Present theories fall into two categories: (1) movement of solute molecules taking place in, through, or upon the surface of sieve-tube protoplasm, and which results from protoplasmic activity; (2) mass flow of solution through sieve tubes or phloem, related, at least indirectly, to activity of photosynthetic tissues, and not dependent upon the activity of the sieve-tube protoplasm.<sup>1</sup> Both mechanisms are based upon ringing experiments which demonstrate limitation of primary movement of foods to the phloem, and upon analytical data which indicate concentration gradients between regions of synthesis and utilization.

Evidence for the protoplasmic streaming hypothesis has been reviewed by CURTIS (22; also 9 and 25); and MASON and PHILLIS have recently elaborated an activated diffusion theory (45, 46, 47, 48) to explain solute movement via the sieve-tube protoplasm. These workers postulate an independent movement of various solutes resulting from independent gradients; they call upon vital activities or surface phenomena to explain the acceleration required.

This paper presents the case for mass flow, drawing attention to certain quantitative evidence and to cytological studies on the activity of sieve-tube protoplasm.

Concentration gradients of organic solutes in the phloem have been adequately demonstrated.<sup>2</sup> That they cause an effective diffusional movement of the solutes is less certain. Physical aspects indicate that diffusion, over long distances, is naturally a slow process; and acceleration of unilateral solute movement, to the required rates, independent of the solvent, would necessitate an immense expenditure of energy. MASON and PHILLIS (48) have recognized this fact.

The mass-flow hypothesis involves a mechanism for transforming concentration gradients into pressure differences. The essential features of this mechanism are apparently incorporated in the normal structure of plants (11, 50). Exudation studies (12, 15, 50, 51) indicate a maintained

<sup>1</sup> HUBER has recently proposed a somewhat similar classification (HUBER, BRUNO. *Hundert Jahre Siebröhren-Forschung*. *Protoplasma* 29: 132-143. 1937.).

<sup>2</sup> See also HUBER, B., SCHMIDT, E., and JAHNEL, H. *Untersuchungen über den Assimilatstrom*. I. *Tharandt Förstl. Jahrb.* 88: 1017-1050. 1937.

pressure gradient in the phloem; and studies on virus movement by BENNETT (2, 3, 4) and others, which indicate a marked parallelism between translocation of virus and organic foods, cast doubt on the independent movement of solvent and solutes. Those who favor mass-flow question the evidence for independent movement of various solutes; and they present anatomical, physiological, and cytological data which indicate (1) that the total phloem (or sieve-tube strand) is the unit of conduit, (2) that mature sieve-tube protoplasm is in a state of reduced activity, and (3) that phloem exudation occurs at rates sufficient to explain common phenomena of growth and storage.

Since the perfect experiment for demonstrating which mechanism actually functions in the phloem has not been devised, judgment must rest, for the present, upon a reconciliation of the recognized facts with known plant structure and behavior. With this in view, new observations on the properties of sieve tubes will be presented, and an attempt made to interpret current research on food movement.

### Phloem studies

Many observations emphasize the important relation between ontogeny and function of the phloem. Previous studies (12, 13, 14, 15) have indicated the changes in form and physiology that take place as the sieve tube matures, passes through its functioning period, and is obliterated. Recent observations confirm these earlier reports.

Young sieve-tube elements are spheroid or cylindrical and well rounded. Having nuclei, slime bodies, and cytoplasm in a high state of activity, they react to stains and plasmolyzing solutions as normal vacuolated living cells. Attending the nuclear disintegration, loss of slime bodies, and alteration of permeability that occurs with maturity, are morphological changes of profound significance (12, 13, 14, 15). At maturity the side walls of sieve tubes are slowly pressed in by surrounding cells so that the elements become funnel-shaped at each end, the transverse area in the center becoming progressively smaller. Finally the walls are pressed together, and the lumen disappears completely; the only structures left at obliteration are the sieve plates, often found lodged between the phloem parenchyma cells. ESAU (26, 27, 29) has illustrated this process of sieve-tube obliteration in the formation of the bundle cap in sugar beet and celery. Though specific studies have been made on only a few species, this general behavior of sieve tubes is well known and has been observed by plant anatomists since the pioneering studies of the past century. It apparently provides almost indisputable evidence that the sieve tube becomes permeable, loses turgor, and is finally collapsed by the pressure of surrounding phloem parenchyma cells (6, 27).

ESAU (29), studying several plants, has noticed that when phloem tissues



are killed by reagents which plasmolyze living cells, young sieve tubes always show shrinkage of the cytoplasm, indicating that they were plasmolyzed in the killing process before the tissue was sectioned. Mature sieve tubes, on the other hand, do not respond to these reagents in this way. As she has pointed out, STRASBURGER made the same observation years ago.

Another unique feature of mature sieve tubes is that the plastids, instead of being associated with the peripheral cytoplasm, are free, floating in the vacuoles (13, 14). This fact again indicates an altered vitality of the cytoplasm, since in all normal living cells the plastids remain attached in some way. The separation of the plastids from the cytoplasm occurs at maturity, when the inner bounds of this layer appear to fade and become indefinite (13).

These many observations, which involve intact as well as sectioned material and which in part date back to the earliest studies on phloem anatomy, indicate with increasing conviction that sieve tubes undergo fundamental changes in their osmotic relations as they reach maturity and are finally obliterated. According to the most logical interpretation, they lose their property of semipermeability and are slowly crushed by surrounding parenchyma cells that survive them by an appreciable period of time. Persistent differentiation and ontogeny of tissue are characteristic of the phloem, and so long as life remains in a plant a certain number of maturing sieve tubes are available to serve their function in conduction.

PHILLIS and MASON (54) object that the highly permeable condition of the mature sieve tube may be an artifact resulting from sectioning. More recently, MASON and PHILLIS (48) state: "In order to insure plasmolysis of the sieve tube it is necessary to carry out the operation on the bark *before it is cut* and removed from the plant." They do not explain how they arrived at this conclusion; and, since the necessary microscopic observation would require some dissection, one cannot easily understand how the work could be done critically. To be certain of his observations one should carry out the plasmolysis under the microscope, where the condition of the sieve tube before and after treatment could be observed. Only in this way can one determine the ontogenetic state of the tube, its freedom from injury, and its reaction to the treatment.

CURTIS objected, several years ago, stating that the permeable condition of the mature sieve tube might be an artifact, but he was unable to substantiate his hypothesis by experiment. Although this point cannot be disregarded, one should remember that the living protoplasm of young sieve tubes and of phloem parenchyma cells is not seriously altered by the injury attending sectioning. Protoplasmic streaming, vital stain accumulation, and plasmolysis occur in these cells despite the sectioning process. These cells are connected by protoplasmic connections as are mature sieve tubes.

There is at present no reason for assigning a special sensitivity to mature sieve tubes. Certainly the evidence indicates lowered rather than increased activity and responsiveness of these cells to injury or manipulation.

Slime plugs in sieve tubes have been studied since the earliest investigations of phloem and have been cited as evidence both for and against a mass flow. Recent observations have explained many of the difficulties in interpreting the relation of slime plugs to translocation. According to a late paper (15), although such structures may be found lodged against the distal sides of sieve plates for several centimeters from the cut end of a cucurbit stem, removal of a thin slice 1 mm. or less in thickness would cause phloem exudation to be resumed. Slime plugs so located are particulate in structure and consist of the decomposition products of the slime bodies and nuclei. An earlier publication (12) cites instances where slime plugs were formed in different regions in sieve tubes by treatment with heat, alcohol, and other coagulating agents. Such plugs are usually amorphous. They consist of the normal sieve-tube sap, which can be coagulated either inside or outside the sieve tube by the same agents.

The particulate slime plugs therefore indicate a mass flow of solution through the sieve tubes, being composed of material filtered out at the plates. Studies on these structures and on the nearby sieve plates give no indication of perforations within the protoplasmic strands traversing the plates. The amorphous plugs are composed of coagulated sieve-tube sap; and according to studies recently reported (15), this sap may continue to flow from cut stems for periods of 24 hours, coming from great distances along the stem. *The sieve tubes must be permeable to this sap, or it could not continue to flow from cut stems for such periods.*

MASON and PHILLIS (48) question the source of the phloem exudate from cucurbits. Repeated studies under the dissecting binocular prove that it comes from the phloem, and with high magnification and well-developed stems it appears to flow from cut sieve tubes. If it does not come from sieve tubes, then the phloem parenchyma must also have some special organization allowing a ready flow of "sugars, nitrogen, phosphorus, potassium, magnesium, and chlorine" and water across them (48). The plasmolytic properties of these cells do not indicate such an organization.

Much confusion has resulted because this coagulable material does not occur in all sieve-tube sap. Products of slime-body and nucleus disintegration, on the other hand, occur in all primary sieve tubes at certain stages in their ontogeny and may in their final stages of disintegration approach (for instance, in the potato plant) the amorphous form of the coagulable sieve-tube sap of cucurbits and similar plants. Unless, however, this amorphous material can be demonstrated in mature elements and coagulated outside the sieve tubes, it must be considered only transitory, and not an essential part of the plant-nutrient supply.

The presence or absence or location of slime plugs depends, therefore, not alone on translocation but also upon the stage of sieve-tube development, the species of plant, and the agents used for demonstration. Without a full knowledge of these matters one cannot correctly interpret the relation of slime plugs to translocation.

Dissection studies on cucurbit phloem reveal some important properties of sieve-tube cytoplasm. Whereas the cytoplasm of phloem parenchyma in this tissue is fragile and easily torn and fractured, the peripheral layer of protoplasm in the sieve tubes is tough, ductile, and somewhat elastic. As seen in dilute water-blue solution, the protoplasmic sheath of mature sieve tubes can be drawn out to many times the length of the element in which it originally occurred. When released it rapidly shortens, but not to its initial length. When stretched to the breaking point it often tears loose from the sieve plate and comes away as a long threadlike strand.

By removing a tangential slice from an intact squash stem, laying bare the phloem, one may observe by reflected light the coagulation of the sieve-tube sap in the tubes. This occurs most rapidly in the cut elements but takes place slowly in deeper layers. Obviously, such a coagulation within the interstices of the phloem walls would increase resistance immensely and stop flow.

If uncut sieve-tube elements are quickly flattened with the dull edge of a scalpel, the sieve plates are deformed, the cupped shape being reversed if the pressure is so increased on the convex side. This fact indicates resistance to extremely rapid flow. The sieve plate of the succeeding element shows little response, however, to this rapid change in pressure; and plates two or more elements away show no reaction. Particulate material in the separate elements does not pass the plates but is filtered out. These observations are further evidence for the relative permeability of mature sieve tubes to dissolved substances.

In connection with the measurements of phloem reported in the following section, a few studies were made on cotton sieve tubes. In common with other species examined, the young sieve-tube elements accumulate neutral red and plasmolyze with strong sucrose solution; but mature elements cannot be so plasmolyzed. Slime bodies were observed in young elements; but these disappeared, along with the nuclei, at maturity. Plastids in the maturing elements may contain several starchlike grains that stain wine red with iodine. In older elements the small starch-grainlike bodies occur free within the vacuole, agitated by Brownian movement. Each sieve-tube element of the *Acala* cotton plants studied contained a small but conspicuous body, having an amorphous core and a thick coating with oddly pitted sculpturing. The phloem parenchyma cells of the stems and leaves had plastids containing chlorophyll in low concentration.

The evidence presented above, though largely circumstantial, indicates that the sieve-tube protoplasm undergoes some marked changes during maturity and that the function of the phloem is most probably involved.

The next studies are concerned with the quantitative relations of the "activated diffusion" hypothesis. Although the Trinidad workers<sup>3</sup> have produced a large volume of data concerning the gross responses of cotton plants to ringing and other similar manipulations, it does not follow that these data substantiate the "activated diffusion" theory. In fact, their own calculations on rates (41, 42), so widely quoted, may not be in accord with these data.

### Quantitative aspects of the "activated diffusion" hypothesis

By rather devious reasoning the Trinidad workers have concluded that solutes move through a "relatively stationary medium" (44) in the sieve tubes by a process termed "activated diffusion" (45). In several places they compare this with polar concentration or absorption across protoplasmic membranes. They point out that both processes depend upon the metabolic activity of cells and utilize energy in performing work. They fail, however, to consider that one is taking place over centimeters and even meters, the other only across microns. Although energy is expended in transferring ions or molecules from the exterior to the vacuole of a cell, or from vacuole to vacuole against a concentration gradient, diffusion is moving these materials through walls up to the cytoplasmic membrane and in most cases probably acts as a limiting factor in controlling rates.

The problem of longitudinal movement through sieve tubes is entirely different. MASON and MASKELL give rates as high as 0.6423 gm. per square centimeter per hour for movement along what they term "sieve tubes" (42, p. 629). Normal rates vary from 0.14024 (41, p. 247) to 0.2356 (42, p. 629). Now, these rates are based on "the whole area of the sieve-tube groups in the phloem, and include companion cells as well as the walls of the tubes" (41, p. 248). Evidently the total transverse area of the inner phloem has been used for computing these values.

Measurements recently made on the phloem in stems of Acala cotton indicate the relation among areas of phloem walls, companion and parenchyma cell lumina, sieve-tube lumina, and sieve-tube cytoplasm. A stem having a transverse area of 2.74 cm.<sup>2</sup> was used, measurements being made on the bark at a point about 4 inches above the soil level.

The bark of cotton consists of groups of phloem, surrounded on all but the cambial side by parenchyma cells of the rays and cortex. The phloem groups consist of intermittent bands of sieve-tube-containing groups and fibers. Fresh sections of these tissues mounted in water were projected

<sup>3</sup> MASON and his associates.

and traced on paper. Then the sieve-tube groups were drawn under the camera lucida, and the drawings and tracings were measured by cutting out and weighing the various areas. Table I shows measurements on tissues and regions of the stem and peduncle.

An oil-immersion lens with critical illumination, used with an ocular micrometer, showed the cytoplasmic layer of the sieve tubes to be approximately  $0.2\mu$  thick. By means of this value and the average sieve-tube areas found (table I), areas for the sieve-tube cytoplasm were calculated. These values, expressed as areas and also as percentages of the actual sieve-tube areas and "sieve-tube-group" areas, appear in table II. A weighted average of the latter values turns out to be 0.98 per cent. Apparently, therefore, the sieve-tube cytoplasm occupies about 1 per cent. of the total area of the sieve-tube groups used by MASON and MASKELL as a basis for calculating rates.

TABLE I

MEASUREMENTS SHOWING RELATIVE AREAS OF VARIOUS TISSUES AND REGIONS IN COTTON BARK. THE TERM "SIEVE-TUBE GROUP" IS USED IN THE SENSE OF MASON AND MASKELL AND INCLUDES ALL PHLOEM EXCEPT THE FIBERS

TISSUE	AREA	PERCENTAGE OF TOTAL
	CM. <sup>2</sup>	STEM
Bark .....	0.766	27.8
Phloem .....	0.314	Percentage of bark 40.8
Cortex .....	0.452	59.2
Sieve-tube groups .....	0.180	Percentage of phloem 57.3
Fibers .....	0.134	42.7
SET 1: 4 PHLOEM GROUPS WITH 66 SIEVE TUBES, FROM THE ABOVE STEM		
	cm. <sup>2</sup> × 10 <sup>-4</sup>	Sieve-tube groups
Sieve-tube lumina .....	0.98	13.7
Companion and parenchyma cell lumina .....	3.97	55.1
Phloem walls .....	2.24	31.2
SET 2: 7 PHLOEM GROUPS WITH 62 SIEVE TUBES, FROM THE ABOVE STEM		
Sieve-tube lumina .....	0.76	9.9
Companion and parenchyma cell lumina .....	4.82	62.5
Phloem walls .....	2.12	27.5
SET 3: 3 PHLOEM GROUPS WITH 172 SIEVE TUBES, FROM PEDUNCLE		
Sieve-tube lumina .....	0.71	10.9
Companion and parenchyma cell lumina .....	3.31	51.5
Phloem walls .....	2.42	37.5

It seems inconceivable that the accelerating mechanism postulated by these workers can act at any great distance from the sieve-tube cytoplasm. Sugar movement along the cytoplasm must take place, therefore, at rates in the order of 100 times those quoted if based on the total area occupied by protoplasm in the sieve tubes. If 50 per cent. of the area is occupied by the permanent cytoplasmic structures and by water of hydration (80 per cent. would probably be a more rational value), then obviously sugars

TABLE II  
AREAS OF SIEVE TUBES AND SIEVE-TUBE CYTOPLASM IN COTTON BARK

SET NUMBER	AVERAGE SIEVE-TUBE AREA	AVERAGE AREA SIEVE-TUBE CYTOPLASM	CYTOPLASM AS PERCENTAGE OF SIEVE-TUBE LUMEN	CYTOPLASM AS PERCENTAGE OF SIEVE-TUBE GROUP
	$\mu^2$	$\mu^2$	%	%
1 .....	148.4	8.4	5.7	0.78
2 .....	122.6	7.8	6.4	0.63
3 .....	41.3	4.5	10.9	1.19
Weighted average for 300 tubes .....				0.98

must move at rates of 200 (to 500) times those originally proposed by these workers (41, 42). The mechanism of VAN DEN HONERT (63) and the suggestions of CLEMENTS (8) and LEONARD (33) are open to similar quantitative criticism.

The work so far described indicates that the protoplasm of mature functioning sieve tubes has unique characteristics. Apparently it exists in a low activity state, being relatively permeable, without streaming motion, and of a tough, ductile nature.

The quantitative interpretation just presented shows the extreme linear rates of unilateral movement necessary to provide for known translocation, and indicates the inadequacy of a purely diffusional type of transport.

In the final analysis, the protoplasmic theories all depend upon a supposed independence of movement of various solutes in the phloem.

Since organic materials are commonly synthesized in localized regions and subsequently moved, obviously gradients in the various forms must occur. But the mere existence of a concentration difference between two plant tissues or organs does not prove that translocation is occurring or normally does occur between them (33, 36, 39). In the translation of concentration gradients to actual movement over any distance, MÜNCH's mechanism (50, 51) is particularly significant, for only where a physical mechanism exists can gradients be related to rapid movement.

CURTIS (22) and MASON and PHILLIS (46) question MÜNCH's mechanism because it will not explain simultaneous movement of solutes in opposite

directions through the phloem, a process which they claim to have demonstrated. The evidence presented for this simultaneous movement is not, however, particularly convincing.

### Evidence for independent movement

CURTIS interpreted his early experiments (17, 18, 19, 20) as indicating a simultaneous upward movement of nitrogen and downward movement of carbohydrates through the phloem. His selection of the mechanism of DE VRIES—namely, diffusion through the phloem accelerated by protoplasmic streaming—to explain movement of all solutes seems to have resulted from this interpretation. Apparently he did not consider, at the time, that a mass flow of organic solutes, including organic nitrogen compounds, might have been taking place upward through the phloem (22, p. 56). His own data, showing large differences early in the season, when phloem movement would have been predominantly upward, and smaller differences later, point to this very possibility. In none of these experiments did he prove a downward movement of carbohydrates.

CLEMENTS and ENGARD (10) have shown that nitrogen absorbed from the soil can move into the top of the plant through the xylem (7), and CURTIS failed to demonstrate complete stoppage of nitrogen movement by ringing. His experiments, therefore, do not prove independent movement of nitrogen and carbohydrates in the phloem.

Recently the Trinidad workers have attempted to demonstrate independent movement of solutes through the phloem by special experiments (44, 46). Using mass analysis of leaves of ringed and unringed cotton plants and testing for nitrogen and carbohydrates in shaded and unshaded leaves, they tried to show a simultaneous movement in opposite directions through petioles and stems. Their first experiment failed, both solutes moving together. They did prove, however, that the movement of these materials could be reversed. Their second experiment showed that xylem movement will adequately explain the upward transport of mineral nitrogen. Though there is some question concerning their determination of carbohydrates by difference between two variables, in the study on movement of stored nitrogen (third experiment) a more serious weakness is the time interval between samplings. Their own earlier work indicates the effect that ringing may have upon xylem transport after an initial period of about 12 hours. Their evidence for independent movement in the stem in this experiment is very inconclusive. The results, furthermore, are subject to a completely different interpretation, a weakness that they fully recognize (44, p. 50).

In their final experiment MASON and PHILLIS (46) attempted by shading and ringing to demonstrate a simultaneous movement of carbohydrates and

storage nitrogen through the phloem of cotton. Their results show a net gain in carbohydrates and a loss of nitrogen in shaded as compared with illuminated portions of single plants and show also that these changes were prevented by a ring between the respective regions. To get significantly different changes, however, they made their sampling periods all 48 hours or more in length; and apparently they failed in their interpretations to recognize the possibility of a diurnal reversal of pressure flow through the phloem.

In their earliest experiments with cotton, MASON and MASKELL demonstrated a diurnal variation in translocation rate. Subsequently they have shown that movement is polar with respect to concentration gradients within the phloem and that by reversing the gradients one can reverse solute movement. In the study under consideration (46) photosynthesis would predominate during the day, causing a downward movement of carbohydrate from the illuminated leaves to the darkened ones. These, being low in carbohydrate, would accumulate it as long as movement continued. When photosynthesis stopped and the gradient flattened, the use of carbohydrates in respiration would allow the pressure to drop to a place where the gradient of hydrolyzed nitrogen compounds in the shaded leaves would cause a slow reversal of flow such as has been demonstrated for autumn leaves. Nitrogen would be accumulated by the high-carbohydrate leaves above. The experiment as performed could not have been more perfectly designed to demonstrate this effect, and such an interpretation seems as logical as that of the authors.<sup>4</sup>

Repeated attempts by able workers have not yet adequately demonstrated independent movement of solutes in the phloem. Some experiments performed (44) have, in fact, shown the opposite, namely, that when carbohydrates and nitrogen move simultaneously in the phloem they go in the same direction and that when they go in opposite directions they do so in separate tissues (44). Virus studies (2, 3, 4) substantiate this observation.

In this connection, SHUMACHER (60) found in studying *Cuscuta* that fluorescein introduced into the phloem of the host was taken up by the parasite with sugars and other foods and that these materials moved together in the phloem of the parasite. MASON and PHILLIS found (47) that

<sup>4</sup> GUSTAFSON and DARKEN failed to prove independent movement; upward movement of phosphorus was slow, simultaneous downward movement of carbohydrate was not measured. GUSTAFSON, FELIX G. and DARKEN, MARJORIE. Further evidence for the upward movement of minerals through the phloem of stems. *Amer. Jour. Bot.* 24: 615-621. 1937.

PALMQUIST failed to prove fluorescein moved at rates exceeding protoplasmic streaming; carbohydrate movement was likewise not accurately measured. PALMQUIST, EDWARD M. The simultaneous movement of carbohydrates and fluorescein in opposite directions in the phloem. *Amer. Jour. Bot.* 25: 97-105. 1938.



not only sugars but also phosphorus, potassium, magnesium, and chlorine respond to influences affecting movement in the phloem. Calcium was immobile after its primary movement to the leaves through the xylem, most likely because it combined with oxalate to form the crystals common in the phloem of cotton.

Until independent movement can be shown, all the critical experiments on translocation can be more adequately explained on the basis of unidirectional flow through the phloem or xylem. To explain pressure flow through both xylem and phloem, furthermore, we have at hand mechanisms that are fairly compatible with present physical concepts.

### Discussion

The diversity of opinion on the mechanism of food movement in plants results from several causes. In going through the immense volume of data available on the subject, one is impressed by the differences in interpretation that occur. Although the grosser responses of plants have been well demonstrated, there is an obvious gap between the experimental work and the final conclusions. It seems timely to consider briefly some of the reasons for this.

### EXPERIMENTAL MATERIALS

In translocation studies a wide variety of plants have been used; and, as a rule, generalized interpretations have not attached sufficient weight to inherent differences occurring among them.

Except for certain horticultural varieties, trees in general grow slowly in relation to their mass. Spring vegetation may be largely produced from stored reserves; summer growth is nourished from current supplies; and products of assimilation may be stored largely during the latter part of the season.

With the herb, vegetation starts from reserves in the seed but soon becomes self-supporting. In many species materials may be stored in specialized organs; in others the bulk of assimilated products goes to produce the plant body. Since these different behaviors make different demands on the vascular organs, translocation must proceed at vastly different rates. Besides, plants of like form may vary widely in the types and amounts of materials moved and stored, and in the rate and reversibility of movement.

Although materials must move great distances in trees, related processes go on slowly; rapid translocation probably does not occur. In vegetative herbs such as tomato and tobacco (14), whose stems and even fruits may contain chlorophyll, products of assimilation are largely used locally; again high rates of movement are not required. For critical studies involving

changes, rates, and gradients, specialized plants having rapid translocation should be used. For such studies the potato (13), the yam (40), the sugar beet (5), and certain cucurbits (12, 15) are favorable. Although not all studies need be limited to these plants, where diurnal variation and incipient changes require frequent sampling to obtain significant differences such plants should be included in any comprehensive work. Processes like xylem and phloem exudation from cucurbits and translocation into potato tubers, beet roots, and cucurbit fruits take place at high rates and are subject to accurate measurement over short time-intervals. Data on these processes should be critical. To state that they are not normal is to beg the issue. Rates of movement in such plants are necessarily high. If interpreted with due regard for the experimental technique involved, they should give a clear picture of the processes at their upper limits.

Though plants may vary widely with respect to rates of movement through the phloem (4, 11, 12, 13, 15, 41, 42) there is, however, nothing in the structure of different forms to indicate that different mechanisms of movement are involved, as suggested by CURTIS (22, note at foot of p. 79). Plant anatomy affords strong evidence that the underlying mechanics of translocation in both the xylem and phloem are the same in all species. Variations in behavior apparently relate more directly to differences in rates of synthesis, translocation, and utilization than to differences in the mechanics of movement.

#### PRIMARY AND SECONDARY MOVEMENTS

Another source of difficulty is the fact that movement of various materials is taking place simultaneously in different tissue systems within the plant body and is activated by different mechanisms. Unless the distinct nature of these is recognized, interpretations may be confused.

Although secondary processes of translocation are often intricate and difficult to study and visualize, certain primary functions are obvious. Mineral nutrients, for instance, are absorbed by the roots and are ultimately distributed throughout the plant. Organic compounds synthesized in green tissues are subject to the same general distribution. In considering translocation, especially with relation to gradients, one should not overlook these facts.

Besides primary movements there is evidence for others. To a certain extent in all plants and very largely in woody plants, absorbed nitrogen combines with organic material soon after entrance to form amide, amino, and other reduced-nitrogen compounds; and nitrogen may move largely in these forms, organic radicles being retranslocated to the foliar regions. As CURTIS and others (22, 33) have pointed out, there seems little justification for MASKELL and MASON's assumption that "the bulk of the mineral

nitrogen absorbed by the roots is carried in the transpiration current to the leaves, where it is elaborated . . . and exported through the sieve tubes back to the roots" (37). On the other hand, as MASON and MASKELL (43) have shown, nitrogen, potassium, and phosphorus may move out of leaves and be distributed throughout the plant via the phloem. Hence secondary movements of both organic and inorganic nutrients may be important in the general process of translocation, the relative rôle of each depending upon rates of absorption, synthesis, and utilization.

A problem often overlooked is the lateral distribution of nutrients from vascular tissues. Since the ultimate distribution of all materials is generally throughout the plant body (30), all nutrients translocated but not used by the vascular tissues themselves are distributed laterally from these tissues; and since the nutrients are largely used by meristems to produce new tissues, processes of growth and translocation are necessarily closely related. Nutrition of meristems is an important function of vascular tissues; and the supply of nutrients to growing cells is apparently not a mere "leakage," but an active movement carried on by the intervening protoplasm. A dynamic equilibrium should exist between the cambium and solutions moving in the adjacent xylem and phloem tissues, growth being conditioned largely by the balance and supply of foods.

Although growth and translocation are closely interrelated, the latter providing food for cell expansion and wall formation, and although growth fixes foods in non-mobile and less active forms so that the growing tissues act as "sinks" for all foods, there is seemingly no evidence that foods are moved for any great distance by the growth process or move through growing cells (56). Differentiation and growth are continuous functions of active phloem tissues; within the phloem there are maintained constantly a number of mature sieve tubes. That growing sieve tubes are active in translocation does not, however, necessarily follow. According to our present knowledge, conduction should be assigned to the mature elements, growth being an effect rather than a cause of the movement and supply of foods (56).

#### PROTOPLASMIC CONDUCTION

Turning now to the actual paths of movement, there are apparently two mechanisms involved in the final distribution of foods. First, there is a slow movement along the cytoplasm from the chlorenchyma to the phloem, and again from the phloem out to the final destination in meristems, storage, or other utilizing cells (13). This is pictured as a diffusional movement accelerated by protoplasmic streaming and wherever the longitudinal rate of transport does not exceed that of protoplasmic streaming it seems that this mechanism suffices. The rapid longitudinal transport of foods at rates of 20 cm. per hour or more is a different sort of process and requires a

different mechanism. Such movement must occur principally in the sieve-tube lumina.

SCHUMACHER considers that his experiments with fluorescein (59, 61) prove a rapid movement of this dye through sieve-tube cytoplasm. There are two other possible explanations of his observations. In 1932 the writer suggested (12) that eosin in SCHUMACHER's early experiments (58) was being absorbed into and carried along the xylem, reentering the phloem at points removed from that of its original application (12, p. 195). RHODES presents a similar interpretation of fluorescein movement in a recent paper (57; see also 47), but SCHUMACHER in repeated experiments, including some in which the xylem from *Pelargonium* petioles was removed, seems to demonstrate that fluorescein may move in the isolated phloem (61).

Granting that fluorescein moves rapidly in the phloem (59, 61), without some definite information on the partition of this dye between the cytoplasm and vacuole it is questionable to attribute the actual transport to the cytoplasm. There is still some doubt as to the localization of fluorescein in the sieve tubes in SCHUMACHER's experiments (59). One might assume a rapid movement of the dye in low concentration through the vacuoles with adsorption by the cytoplasm to explain his observations. Other dyes, such as aniline blue, are accumulated in the cytoplasm of mature sieve tubes from a low external concentration. Regardless of the path of movement of fluorescein, however, sugars, which move in vastly larger amounts through the phloem, would have to overcome extreme resistance to travel at the required rates through cytoplasm.

Continued experiments on chilling and coating petioles (21, 24, 47) have failed to prove that sieve-tube protoplasm plays an essential rôle in longitudinal transport. Reduction in rates may be explained on the basis of viscosity change and altered permeability of phloem tissues. This type of experiment is of doubtful value in the final solution of the problem.

According to MASON and PHILLIS (47, p. 497), "The mechanism activating diffusion (in the sieve tube) consists of some special organization in the cytoplasm, maintained by metabolic energy, whereby the resistance to solute movement is so reduced that materials diffuse in the sieve tube at rates comparable with those in a gas." For reasons unknown, these writers make an illogical choice in selecting the solute as the object of this special mechanism. Experiments on water and solute absorption, plasmolysis, and water movement in tissues indicate that the solvent, water, moves through protoplasm more readily than do solutes. If water is included among the substances that move by "activated diffusion," then there is little difference between the "activated diffusion" and the pressure-flow theories, for movement in the latter instance is a special case of diffusion of solvent and solute through tissues along a concentration gradient of solution. Such flow must

occur through the walls of root hairs, possibly through the vacuoles of endodermal cells, and through the walls of xylem vessels in roots that are absorbing water and solutes simultaneously. The apparent independence of solute movement under these conditions might result from differential resistance to various solutes by the membranes traversed, such resistances being related to molecular sizes, hydration, and the like. Whereas the pressure-flow theory pictures acceleration in the sieve tube as resulting from a hydrostatic pressure, developed osmotically and exerted impartially upon solute and solvent, the "activated diffusion" theory requires a selective application of energy to the solute molecules by the protoplasm. The former is based upon almost innumerable observations of phloem exudation where solute and solvent flow from cut phloem in volumes entirely too large to have come from the cut elements alone (15). The latter is entirely hypothetical and rests largely upon the questionable evidence for independent movement that was considered in a previous section.

SCHUMACHER, discussing the movement of fluorescein in cucurbit (59), states that he plasmolyzed sieve tubes with a sucrose solution. Evidently he was observing young sieve tubes. His studies on exudate, however, must have involved mature elements. Apparently he overlooked the importance of sieve-tube ontogeny in this problem and consequently misinterpreted many of his observations.

#### MOVEMENT ALONG CELL WALLS

MASON, MASKELL, and PHILLIS have objected to my postulation of flow along phloem walls. Although I have altered my views on this subject (15), it still seems probable that movement through wall material takes place (1) from the furthest differentiated sieve tubes to the growing points of root and shoot, (2) across sieve plates from element to element, and (3) laterally when gradients are altered by such practices as partial ringing (42) and sectional pruning.

Considering for the moment that when a healthy plant cell is plasmolyzed with sucrose, the molecules may diffuse through the wall in a matter of minutes, whereas they may take hours or even days to traverse the cytoplasm, flow along walls does not seem irrational compared with movement at even higher rates through cytoplasm. If the cytoplasm of mature sieve tubes is permeable (13), this objection is less pertinent. It seems highly unlikely, however, that protoplasm devoid of streaming movements and completely permeable to solutes could be the seat of such high metabolic activity as would be required to provide the necessary acceleration. Rather, protoplasm of the functioning sieve tubes seems to be definitely degenerating and passing into a condition of prolonged senility, followed by death (12, 13).

## INADEQUACY OF MASS ANALYSIS

Many interpretations by the Trinidad workers and others indicate the inadequacy of mass-tissue analysis for studies on translocation. CURTIS has pointed out this weakness (22). Granted that photosynthesis occurs in the plastids and that movement of carbohydrates to the sieve tubes takes place by diffusion, accomplished via the protoplasm and accelerated by streaming (13, 22, 50, 51), there is apparently no reason to postulate a polar accumulation by transition or companion cells, and secretion into the sieve tubes (53). One might more logically assume that carbohydrates are high in concentration in the cytoplasm of mesophyll cells and move along a positive gradient into the sieve tubes. Mass analysis gives predominantly a picture of vacuolar concentrations and indicates simply the amount of solutes maintained in the vacuoles of mesophyll and cortical parenchyma cells to preserve their form and to insure them against desiccation. Vacuolar concentrations in the sieve tubes are in equilibrium with those of the cytoplasm because of the peculiar permeability conditions; and there alone may the true status of nutrients within the actual paths of movement be measured. Until methods are developed for determining sugars in the cytoplasm and in sieve tubes, it seems futile to interpret mass analyses in terms of translocation. LOOMIS's assumption of polar movement of carbohydrates in maize (35) is based upon such faulty interpretation. If one does not assume that food movement determines fertilization, LOOMIS's defoliation and defruiting experiments all fit the mass-flow theory. Gradients as determined by mass analysis reflect vacuolar concentrations in the various tissues analyzed. Although they indicate relations of storage and water loss, they form no adequate basis for the assumption of polar movement.

According to recent experiments on translocation in the sugar beet (5), phloem parenchyma may play an important rôle in maintaining necessary gradients by temporary storage of sugars; and the results reported by PHILLIS and MASON (53) are probably more directly related to this effect. This suggestion, however, should be checked by analyses showing vacuolar concentrations in individual cells.

As this discussion shows, more detailed information on tissues involved in absorption and transport is needed for an understanding of the mechanics of these processes. Mass-tissue analysis gives an excellent picture of the gross responses of plants to ringing (22, 34, 36, 41, 42) and similar practices. Workers, with few exceptions (22), agree that the primary upward movement and distribution of mineral nutrients absorbed by the roots take place through the xylem and that organic materials synthesized in the leaves pass to utilizing cells through the phloem (52). The final agreement upon the mechanics of movement in sieve tubes, however, must await the development of methods for studying single tissues and possibly single cells.

### Conclusions

The translocation problem is obviously complex. At least two tissue systems are utilized in actual conduction; all tissues of the plant and most functions are involved directly or indirectly. Only by viewing the problem in this broad aspect may one hope to integrate its various phases into an organized mechanism and to harmonize it with known plant structure.

Root absorption by higher plants, involving as it does accumulation by single cells, secretion into nonliving ducts, and movement both to and away from the functioning organs (16), constitutes the primary process in the translocation of mineral nutrients and water (31). Absorption and translocation by roots seem, in fact, inseparably related and should be studied as phases of a single phenomenon.

In dealing with the problems of absorption and translocation we cannot neglect structure. There is, for example, no apparent reason for assuming a general upward movement of nitrogen in the phloem, simultaneous with the downward passage of carbohydrates. Root anatomy apparently indicates much greater difficulty in absorption by the phloem than in release into the xylem (11, 16). No mechanism in the phloem explains the extremely rapid rise of minerals observed in the plant. Many reports on the occurrence of both inorganic and organic nitrogen compounds in xylem sap indicate their presence in the water-conducting tracts (1, 55, 62). This general knowledge, with the results of MASKELL and MASON (37, 38), HOAGLAND and BROYER (32), CLEMENTS and ENGARD (10), and others, points to conduction of mineral nutrients in the xylem.

Viewing mineral movement in relation to anatomy and considering the rates and distances involved, one may conclude that the roots are the primary source of minerals in the plant; that the minerals may diffuse laterally from the xylem during their upward movement; and that any residual minerals not held by the leaves may be retransported via the phloem with carbohydrates. The leaves would thus constitute not only a portion of the sink for minerals in primary movement but also the source of the secondary supply. This flow of carbohydrates and residual mineral nutrients in the phloem is apparently the most important supply to the primary-shoot meristems.

In certain herbaceous plants plentifully supplied with nitrogenous fertilizer there may be a large secondary movement of nitrogen out of the leaves. In many plants, however, especially in woody ones, the primary movement would predominate; and the secondary movement might cease entirely, at least during the latter part of the growing season. Evidently a secondary release from most leaves occurs before death or under heavy shade.

Data on problems of translocation are rapidly accumulating. New ob-

servations have been made on phloem exudation (2, 3, 4, 15).<sup>5</sup> Although this evidence is not complete, each added contribution helps. Plants vary widely in their rates and volumes of phloem exudation. In view of their forms and functions, this variation seems rational. Highly vegetative herbs, as pointed out, need not move organic foods over great distances. Trees, because of relatively slow growth over long periods, also have low requirements except in fruit formation. Only plants that rapidly store large quantities of carbohydrate require high rates—for example, cucurbits and sugar beets, which exhibit rapid phloem exudation.

The pressure-flow mechanism should meet certain functional and structural requirements. Turgor gradients pictured by MÜNCH have been proved nonexistent (22, 23, 53). The sugar-beet plant is an excellent example, having 5 to 10 per cent. sugar in leaf cells, about 10 per cent. in phloem exudate, and as high as 25 per cent. in root storage cells. Both the diffusion and pressure-flow theories require, apparently, accumulation within certain receiving cells to concentrations higher than those of the conducting tissues to provide the necessary concentration gradients.

Whereas WEEVERS and WESTENBERG (64) were unable to collect water of exudation from the cambium by the method of MÜNCH, MOLOTKOVSKII (49) was apparently successful. He agrees with MÜNCH that this water comes from the cambium and suggests that the sugar appearing during the first few days comes from injured phloem. Organic materials appearing later in the experiments come from contaminating fungi. The occurrence of this type of exudation probably depends upon a relatively abundant supply of water to the tree. A demonstrable flow is not essential to the pressure-flow theory, for the water accompanying foods in the phloem may be largely used in growth or even lost by evaporation through the bark. True phloem exudation is the most important prerequisite; and where it fails entirely, either translocation is not taking place or some other mechanism is involved. Recently reported xylem-exudation studies show another weakness of the MÜNCH hypothesis. MÜNCH's explanation of root pressure as resulting from unilateral secretion of water from the phloem will not explain the large volumes nor the ion concentrations of exudate from excised root systems. His view of sieve-tube structure has, furthermore, proved untenable, in the light of more recent studies. There seems to be no reliable evidence for intervacuolar passages within the protoplasmic strands of the sieve plate.

As critics of the pressure-flow theory have pointed out, phloem exudation varies among species and may not be demonstrable in some. Demonstration of this phenomenon, however, depends upon several factors. If any appreciable volume is to come from a cut stem, rapid translocation must

<sup>5</sup> See also footnotes 2 and 6.



be in progress, and exudation must exceed evaporation. Capillary absorption by intercellular spaces of the cortex and pith must be prevented. Flow, furthermore, must be rapid compared with stoppage. In order to survive, plants must have developed a mechanism for preventing excess leakage. A recent paper reports observations on this phenomenon (15). The relative rates of flow and stoppage determine the volume of exudate that comes from a single cut. The best known way to maintain flow is to repeat the cutting (11, 12, 13, 15). By proper technique, phloem exudation may eventually be demonstrated in all higher plants.<sup>6</sup>

The physiological-anatomical relations of phloem transport form a fertile field for study. Much should be known concerning the limiting layer that retains, within the phloem strands, materials in transport. Dissection experiments (11) indicate such a layer. Though the relation of virus movement to translocation of foods is being reserved for a later paper, the recent work on curly top should be noted (2, 3, 4, 26). Evidence for movement of virus in the phloem with foods and for exudation of the virus-bearing sap from necrotic phloem through a normally impervious layer seems convincing.

CURTIS has pointed out the difficulties that result from cutting a system composed of nonrigid cells acting under a positive pressure (22). A method for artificially injecting such a system without injury would be highly desirable. As BENNETT's work shows, the inoculation of curly-top virus by the sugar-beet leafhopper provides such a method: though injury results to the phloem, translocation may be rapid enough to carry the virus considerable distances before necrosis becomes serious. On the other hand, the study of internal symptoms has itself proved valuable (28), providing additional evidence for the permeability of mature sieve tubes.

The foregoing discussion should show that the divergence of opinion that has characterized translocation study in the past is narrowing and that lack of agreement results not so much from conflicting bodies of data as from differences in interpretation. Workers supporting both the protoplasmic theories and the pressure-flow mechanism assume a specialization of the sieve-tube protoplasm and call upon the activity of living cells. The basic assumption of the former group seems to be that there exists in the phloem rapid transmission of molecules independent of the solvent and related to a high activity state of the sieve-tube protoplasm.

According to the pressure-flow theory, conduction is permitted by a high permeability of the sieve-tube cytoplasm resulting from a decline in its activity state. Cytoplasm in this condition apparently allows a ready passage of food materials in solution.

<sup>6</sup> See recent experiments of MOOSE. MOOSE, CARLETON A. Chemical and spectroscopic analysis of phloem exudate and parenchyma sap from several species of plants. *Plant Physiol.* 13: 365-280. 1938.

Obviously more research will be required to settle the controversial phases of this problem. It would seem imperative that this research include detailed cytological studies on phloem tissues and that the relation of ontogeny to function be kept in mind.

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UNIVERSITY OF CALIFORNIA  
DAVIS, CALIFORNIA

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# ICE FORMATION AND THE DEATH OF PLANT CELLS BY FREEZING

IRENE H. STUCKEY AND OTIS F. CURTIS

(WITH ONE FIGURE)

## Introduction

The resistance of plant tissues to low temperatures and the causes thereof have been the subject of extensive research by many investigators. Since there have been comprehensive reviews of both phases of the subject written by CHANDLER (4, 5), ROSA (22), ÅKERMAN (1), MAXIMOV (15), LEVITT and SCARTH (13), and SCARTH and LEVITT (23), this paper refers only to those investigations which are specifically related to the immediate problem.

In all but very few of the previous studies of ice formation in living tissue, the material was frozen by various means, and then after thawing, was examined either grossly or microscopically. What had occurred during freezing was postulated from the appearance of the thawed tissue. Of the few investigators who observed freezing directly, apparently MOLISCH (17) was the first to see ice formation as it occurs in living cells. His microscope was put into a specially constructed ice chest with openings to give access to the controls. The apparatus was inconvenient to use, and the temperature could not be regulated, but MOLISCH was able to use it to watch freezing in *Amoebae* and in several plant tissues.

WIEGAND (26) investigated the condition of buds during subzero weather. He used a microscope and sectioning apparatus out-of-doors and noted where ice was formed in the buds. Ice was found as a single continuous layer always in the mesophyll of the leaf or scale, never between the scales. The cells on either side were in a state of collapse and often occupied less space than the ice sheet. Ice formation was observed in some species at  $-18^{\circ}$  C., in others not until  $-26^{\circ}$  was reached. This difference seemed to be determined by the size of the cells and the amount of water which they contained. The ice decreased in amount as the temperature rose and finally disappeared at  $-3.2$  to  $-2.3^{\circ}$  C. No injury was noted in buds in which such ice was formed, even after rapid thawing at  $21^{\circ}$  C.

SCHANDER and SCHAFFNIT (24) used a cold chamber mounted on a microscope stage. The chamber was cooled by passing a jet from a tank of compressed  $\text{CO}_2$  through ether. By this means the object could be quickly cooled to any temperature down to about  $-30^{\circ}$  C., and the frozen tissue could be observed under the microscope. ÅKERMAN (1) used the MOLISCH technique and directly observed the tissues in the frozen condition.

CHAMBERS and HALE (3) made microdissection studies of freezing frog muscle, *Amoebae*, and the epidermal cells of red onion. The operations were performed on prechilled material in a cold room at temperatures ranging from 0° to -5° C.

The methods used by these workers are not entirely suitable for observing the process of freezing. The purpose of this investigation was to find some simpler means for directly observing ice formation in plant tissues, and once this method was established, to apply it to some of the problems concerned with the resistance of plants to low temperatures. We wished to determine more certainly, if possible, where and under what conditions ice occurs, and the relation of water content to resistance.

The observations were made with a Spencer binocular microscope of the type with a square stage and inclined oculars. The square stage was necessary because of the design of the cold stage attached to it. Sufficient magnification was obtained with 10×-oculars; oculars of higher power gave an unsatisfactory image. It was essential to replace the 4-mm. objective of the microscope with one having a greater working distance, consequently the objective used was an achromatic model 4-mm., N.A. 0.65, working distance about 0.9 mm. The top lens of the condenser was removed to raise the point of convergence of the rays of light to the level of the mount in the cold stage. The light source was a microscope lamp with a 250-watt filament bulb, a condenser, and a ground glass.

For the actual freezing process, the cold stage and circulatory system described by MASON and ROCHOW (14) were used. It was found desirable to make a few modifications for best results with biological materials. The bottom cover glass was sealed in place with LePage's cement rather than vaseline. Celotex was used to insulate the stage since sheet cork of the desired thickness could not easily be obtained at the time the stage was made. As stated by the designers of this apparatus, the operation of the system would have been easier if the channels in the stage had been larger, and the circulatory tubes of correspondingly greater diameter. This would allow for greater flow of liquid, and consequently better temperature control. Ether rather than acetone was the liquid used with the solid carbon dioxide in the Dewar flask. A still better method would involve the use of a mechanical water cooler instead of solid carbon dioxide. The plant material was mounted in paraffin oil on an 18-mm. no. 1 cover glass, and over it was placed a fragment of no. 0 cover glass large enough to cover the specimen. It was found easier to mount the material in this way than between two cover glasses. This mount was placed on a tripod of copper wire in the well of the stage. A 25-mm. cover glass which rested on the flange of the stage was placed over it, leaving an air space underneath. The air space insulated the cover glasses, and the objective conducted heat to the well to such an



extent that the material would not freeze when the objective was in focus. To avoid this difficulty, the designers of the stage recommend that the mount be so made that some of the material to be frozen is in contact with the stage; but with the material used, this was not feasible; therefore, solid carbon dioxide was used to chill the objective. The dry ice was packed into the space between the objective and the stage, above the upper cover glass. A piece of sheet asbestos was fitted over this to prevent the solid carbon dioxide from evaporating too quickly. A dissecting needle could easily be thrust through this layer of dry ice to tap the cover glass and prevent supercooling. The air space between the upper cover glass and the one on which the material was mounted gave adequate insulation against the low temperature induced by the presence of the solid carbon dioxide on the upper cover glass. Whenever this cover glass was broken, however, the mount froze immediately; therefore, no temperature readings were considered valid unless the cover glass remained unbroken throughout the freezing.

Exact determinations of temperature were not considered essential for this phase of the investigation. An alcohol-filled, low temperature thermometer reading to  $-50^{\circ}$  C. was inserted in the thermometer opening in the cold stage. This thermometer was calibrated by observing the temperature at which distilled water froze when placed between cover glasses in the same way as the plant tissues were mounted. This temperature was found to agree within  $2^{\circ}$  C. with the temperature as determined by immersing the thermometer in melting ice. There was less error when the temperature change was gradual than when it was rapid. Since the difference in temperatures was always greater than the experimental error, this method was considered sufficiently accurate. For more precise temperature readings it would be desirable to use a thermocouple instead of a thermometer.

To insure a uniform water content in experiments where wheat seeds were used, lots of 100 seeds were placed in 2-oz. bottles with a known volume of water. These bottles were then fitted securely with wooden wedges into holes in a large wooden wheel which was rotated slowly with a small electric motor. The rotation kept the grains constantly rolling over so as to bring about a uniform distribution of water. The seeds were weighed when air dry before the water was added to them, and then again after they had been rotated with the water for a given length of time. Some of the highest percentages of water determined may not have been accurate, since in some of these cases not all the water was absorbed.

For gross freezing, a General Electric water cooler was used. Alcohol, in which the bottles containing the seeds were suspended, was placed in the compartment designed for water, and the temperature was regulated in the usual manner. After treatment, the seeds were germinated in sterilized petri dishes containing layers of moist paper toweling.

### Observations

For those studies involving microscopic observations, isolated cells of *Sedum acre* L., stamen hairs of *Zebrina pendula* Schnizl., and prothallia of *Polypodium aureum* L. with large irregular plastids were used.

#### ICE FORMATION IN CELLS OF *Sedum acre* L.

The cells of the *Sedum* were obtained as follows: A leaf, split with a dissecting needle, was placed in a drop of paraffin oil on a slide and pressed gently under a cover glass. Then when the cover glass was lifted and the large fragments of the leaf were removed, isolated mesophyll cells, each surrounded by a thin film of cell sap released from the vacuoles of crushed cells, remained in the oil. These cells froze readily, but the thin film of sap froze more quickly than the cell contents and concealed the behavior of the latter.

#### ICE FORMATION IN *Zebrina* STAMEN HAIRS

The *Zebrina* stamen hairs were mounted directly in paraffin oil. The streaming, which at room temperature was fairly rapid, gradually became slower as the temperature dropped, and stopped completely at  $0^{\circ}$  to  $-2^{\circ}$  C. In some instances Brownian movement of the granules began when streaming ceased; it persisted for a few seconds and then stopped. That this was not observed in all cases is probably attributable to the speed at which the temperature was lowered. No visible disintegration of the cells was evident after streaming had stopped. Mature cells froze when the temperature reached  $-7^{\circ}$  or  $-8^{\circ}$  C.; immature cells were more resistant, remaining unfrozen to a temperature of  $-17^{\circ}$  C. These younger cells seemed to have a very high osmotic concentration which apparently decreases rapidly as the cells mature. These younger cells, moreover, showed larger granules and a greater concentration of anthocyanin.

As the freezing point was approached, it was necessary to tap the cover glass with a needle in order to prevent excessive undercooling. Formation of ice crystals always occurred first outside the field of vision, and as each cell froze, beginning at the end toward the base of the stamen hair, it inoculated the one next to it until in rapid sequence all of the cells in each hair had frozen. Each cell as it froze became filled with a closely packed mass of granular ice crystals, and was thereby rendered opaque. Differences in the freezing of the cell parts could not be observed. When cells frozen in this manner were thawed slowly, the cell sap escaped, since the plasma membrane had become more permeable, and the sap collected as droplets in the surrounding paraffin oil. When this occurred, the cells became shrunken and the cell walls very irregular. The cytoplasm assumed a very granular appearance and became stained purple by the vacuolar pigment. The nucleus was much distended and glassy in appearance and more conspicuous than

in the living cell. The appearance of cells after more rapid thawing was similar to that just described; however, in these cells, the cell retained more of its normal semipermeable character, since less of the cell sap escaped, and the cell walls had more nearly their usual contours.

#### ICE FORMATION IN *Polypodium* PROTHALLIA

The *Polypodium* cells, like those of *Zebrina*, showed excessive undercooling unless the cover glass was tapped with a needle. Ice formation took place in these cells in two ways; one of these may be described as "wave front advance" freezing, and the other as "single cell" freezing. In the first type, inoculation occurred outside of the field of the microscope, and from the first crystals seen, the ice front advanced in a series of localized waves, until the entire visible portion of the field had become frozen. The crystals formed under these conditions were compact and granular. In the "single cell" type of freezing, isolated individual cells froze here and there across the field until all the visible ones were frozen. In some of these, a slight quivering of the cell contents took place just as inoculation occurred, giving the impression that the protoplast had a jelly-like consistency. The ice crystals formed in some cells were compact and granular, like those of the "wave front advance" type. The crystals in other cells were needle-like and developed from one or more sides of the cell. These crystals elongated rapidly until they extended across the cell, giving the latter a striated appearance.

In all cases, the chloroplasts froze a few seconds after the other parts of the cell had been obscured by ice. This delay indicates the presence of a membrane around the plastid which may allow greater undercooling to occur. The plastid probably also has a higher osmotic concentration. Some differences seemed to exist between the time of freezing of the vacuolar sap and that of the cytoplasm, the latter freezing first; but the order of freezing could not be determined definitely with the apparatus used. The relative time of freezing of the nucleus remains uncertain.

When the temperature was raised, the crystals of ice melted gradually and formed drops of water. These drops fused and the protoplasts again became visible. The appearance of these protoplasts was comparable to that of the stamen hairs after thawing: the cytoplasm was granular and the outline of the plastids irregular. The plasma membrane likewise became more permeable, as evidenced by the droplets of cell sap accumulated in the oil outside. The cell walls retained their normal contours. No difference was observed between slowly and rapidly thawed cells.

#### INFLUENCE OF SUCROSE AS A PROTECTIVE AGENT

Experiments were performed with sections of the epidermis of red cabbage which had been kept in the cold room at 5° C. for several months and

presumably were in a hardened condition. Strips of epidermis were put into small test tubes, and the tubes were then immersed in an alcohol bath cooled to  $-10^{\circ}\text{C}$ . After the tubes had remained at this temperature for 10 minutes, chilled sucrose solutions of varying concentrations were poured gradually down the sides of the tubes. The sections were gradually brought to room temperature over a period of 2 hours and then examined under the microscope. Most of the cells showed some degree of plasmolysis, but there was no correlation between the extent of the plasmolysis and concentration of the thawing solution. When the sucrose solution was gradually replaced with distilled water, the protoplast disintegrated, the red pigment diffusing into the bathing solution. Even if the sucrose solution was diluted gradually over a period of an hour, in no case did the protoplast remain intact. This suggests that since the tubes in which the strips of epidermis were frozen were at room temperature when the experiment began, the cells became somewhat plasmolyzed by withdrawal of water during the first 2 or 3 minutes that the tubes were in the freezing bath. Then when they reached the temperature of the bath, or some critical point above this temperature, ice was quickly formed inside the protoplast and the cells retained the appearance they had at the time of freezing. The addition of the sucrose had no protective action in delaying deplasmolysis of the protoplast, but it did prevent the immediate disintegration of the cytoplasmic membranes which occurred in tissue thawed in air or water. The evidence suggests that the cells were dead when the sucrose solutions were added, and the "protective" action was purely mechanical.

To check these observations, the experiment was repeated. The test tubes used, however, were first chilled to the temperature of the bath before the strips of epidermis were introduced. The results were the same except that there was no pseudo-plasmolysis. The protoplasts were killed before any water was withdrawn from them.

Similar experiments were performed freezing the tissue in water and in solutions of sucrose ranging from 0.5 M to 4 M. At  $-10^{\circ}\text{C}$ ., the water, 0.5, 1, and 2 M sucrose solutions appeared completely solidified. Ice crystals were formed in the two stronger solutions (3 and 4 M), but there was still a small amount of unfrozen solution present. If the solutions were cooled to the temperature of the bath before the epidermal strips were put into them, no plasmolysis occurred. But if the tissue remained in the solutions for a few minutes before being chilled, plasmolysis took place. When these sections were thawed gradually, and then the sucrose was replaced with distilled water, the protoplast swelled rapidly and the protoplasmic membranes disintegrated. Even when the dilution occurred gradually, the plasma membrane became disorganized and the cell sap diffused out into the bathing solution. In a few strips of epidermis, some two or three very

small cells deplasmolyzed completely and were apparently alive, but when they were plasmolyzed again, they did not survive. There is some doubt as to whether ice was actually formed in these cells, since they were very much smaller than the surrounding cells which did not survive, but even these smaller cells did not survive after the second plasmolysis.

ILJIN (9) found that similar strips of red cabbage epidermis were protected by thawing in sugar solutions. He suggests that the solution absorbs the water which is lost from the cells when the temperature is lowered and prevents its rapid imbibition by the protoplast when thawing takes place. And if the rate of expansion of the cytoplasm is more nearly equal to that of the cell wall, rupture of the plasma membranes and subsequent death of the cell does not occur. ILJIN's experiments may be criticized because the deplasmolysis which was used as the criterion of life was not carried to completion. That the protoplast would expand partially was considered sufficient proof that the cell was alive. The studies of CHAMBERS and HÖFLER (2) with isolated tonoplasts have shown that this membrane can be made to expand and contract with solutions of various concentrations even outside the cell and entirely free from protoplasm. CHAMBERS and HALE (3) describe frozen epidermal cells of onion bulb scales in which the cytoplasm and nucleus had disintegrated as a result of freezing, yet the tonoplast or vacuolar membrane remained intact and showed deplasmolysis on thawing. The evidence presented by ILJIN is not entirely convincing that the cells were still alive after being thawed in the sucrose solutions.

In the present work some of the strips were frozen in sucrose solutions in the cold stage. If the cells were strongly plasmolyzed before freezing took place, the temperature required for ice formation was much lower than if the cells were not plasmolyzed, which is to be expected in view of the higher osmotic concentration within these plasmolyzed cells, and perhaps because of greater undercooling in this condition. When the cells were very strongly plasmolyzed, ice was not formed at  $-10^{\circ}\text{C}$ .

In ILJIN's experiments the temperature was lowered much more slowly than in those here reported. The tissues were kept at some of the higher temperatures for as much as 24 hours. This not only would allow hardening to take place, which would make the cells more resistant to low temperature, but also allowed for plasmolysis which so increased the osmotic concentration of the cells that ice formation was prevented until a very low temperature was reached. This is in agreement with the findings of ÅKERMAN (1).

Experiments such as have just been described were performed with fern prothallia also. The results were comparable.

#### ICE FORMATION, WATER CONTENT, AND COLD RESISTANCE OF WHEAT

Wheat was chosen for the material to be studied because so many of the previous studies of cold resistance and hardness have been made with wheat,

and because the seed coats do not interfere with rapid absorption of water. During the winter of 1935-1936 a local commercial grain was used for most of the tests, and the results were checked with Minhardi. During 1936-1937, Minhardi was used altogether, with the exception of one group of experiments for which a non-hardy wheat, Leap, was obtained from the Department of Plant Breeding.

The percentages of germination of the controls was above 95 per cent. in all the wheat used and the seeds were kept in a sealed jar in a cool room to prevent any marked changes either in water content or in vigor. The grains were hand selected for uniform size and were divided into lots containing 100 seeds each. The weight of these seeds before any water was added was considered the "air-dry" weight. The "oven-dry" weight was obtained after drying samples of seeds in a vacuum oven for 288 hours at 90° C. The water content of air-dry grain was determined at intervals for different lots of seed to check the amount of water they contained, so as to be sure that the grain used over a period of time had the same initial water content. Calculated on this oven-dry basis, the air-dry seed contained 10 per cent. of water. For those lots of seed to which water was added, the percentages were computed from the increase in weight of water over the air-dry weight, the latter being considered as 10 per cent. from the previous determinations. The lots of seeds were weighed, put into bottles and a known volume of water added from a burette. The bottles were then stoppered tightly, put into the wheel, and rotated for 48 to 96 hours. In some of the first experiments, this rotation was performed at 25° C., but since at that temperature, seeds containing more than 31 per cent. moisture sprouted, a temperature of 4° C. was used. At the end of the period of rotation, the seeds were weighed again and the average percentage of water in each lot of seed was calculated. Seeds of each class according to their water content were then exposed to a temperature of -20° to -25° C. for 5 hours. After treatment they were germinated at 25° C. Counts were made at the end of 36 to 72 hours. The wheat germinated very quickly, and only rarely did those seeds which failed to germinate after 72 hours germinate at all.

There is no essential difference between the shape of the curve of germination with the Minhardi wheat and that of the Leap. These results are shown on the graph (fig. 1).

Sections of the endosperm of the wheat kernels cut freehand with a razor were mounted in mineral oil in the cold stage. No freezing could be observed except in those endosperms which were saturated with water. Since the endosperm could not be used, the embryo was tried. One slice was cut from the tip of the embryo and discarded; the next slice was used. These slices were cut freehand as thin as possible. Most of the sections used were in parts only one cell thick; probably they were between 10 and 20

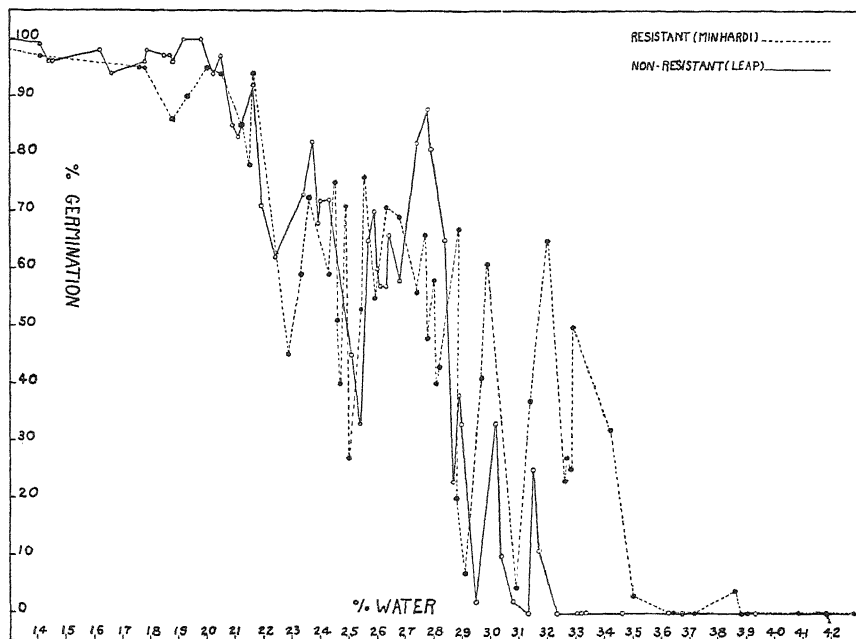


FIG. 1. Comparative behavior in germination of a resistant and a non-resistant variety of wheat at different moisture contents, after subjection to low temperatures ( $-20^{\circ}$  to  $-25^{\circ}$  C.) for 5 hours.

microns thick. No attempt was made here to find the temperature at which the seeds of different water contents froze. The purpose was to determine as quickly as possible whether seeds within certain water-content ranges would freeze or would not freeze. None of the seeds which contained 24 per cent. or less water would freeze even at temperatures down to  $-25^{\circ}$  C. In the intermediate group (water content 27 to 28 per cent.) ice was observed in about half the sections but in the upper range (30 to 31 per cent.) all the sections examined showed ice formation. In some of the sections with 27 to 28 per cent. water, in which no ice was formed, apparently some ice formation took place in the intercellular spaces, but it was very difficult to distinguish between air and water in the intercellular spaces; it cannot be stated positively, therefore, whether or not freezing did occur in the intercellular spaces and not in the cells. When the temperature was lowered the cell contents became darker, perhaps because of some colloidal change or of a difference in the refractive index of the water at the low temperature. This darkening occurred regardless of ice formation. Finally, when the temperature became still lower, ice crystals appeared and the sections became opaque. The same precautions were taken to prevent excessive under cooling as were used with the other materials. To shorten the time for these

determinations, this freezing was done at temperatures lower than was necessary for ice formation. It was not as important to find the exact temperature at which ice was formed as it was to determine as rapidly as possible whether or not the embryos would show ice formation at any temperature. The results of the germination tests are shown on the graph. There was a correlation between the water content of the seeds, ice formation as observed in sections of the embryo, and their resistance to freezing. This relation between water content and resistance to freezing is in agreement with the findings of WHITCOMB and SHARP (25) for wheat, and of KIESSELBACH and RATCLIFF (12) for corn.

#### ICE FORMATION IN HARDENED AND NON-HARDENED WHEAT LEAVES

Plants of Minhardi wheat were grown in pots until they were about 6 inches high. Some of the pots were then placed in a refrigerator at 8° C. every night for ten nights and kept in the greenhouse during the day, while other pots remained in the greenhouse continuously. As far as possible, the soil moisture was kept the same for both sets of pots. ROSA found that cabbage plants exposed to sunlight and rather high temperatures during the day, and to temperatures close to freezing at night became hardened easily. The wheat plants hardened in this way were darker green, had thicker leaves, and were smaller than the actively growing plants. On one occasion, two pots containing 100 plants each, one set hardened and the other non-hardened, were left outside all night when the temperature reached -19° C. The hardened plants were not injured, while the non-hardened plants were so injured that only the roots survived. These plants subsequently produced more leaves when they were returned to the greenhouse. Neither the old nor the youngest leaves on the hardened plants were injured. When examined on the freezing microscope, the leaves from the non-hardened plants showed ice formation within the cells at -10° to -12° C., while the hardened leaves showed it first at -25° to -27° C. The cells were all killed when ice was formed, whether hardened or not. The hardiness was lost in three or four days if the plants were not placed in the refrigerator at night. When the pots were left in the greenhouse for three days, there was almost no difference in the temperature at which ice was formed in the leaves of previously hardened and non-hardened plants.

#### Discussion

Three hypotheses commonly offered to explain the death of plants exposed to low temperatures are that this injury is due to drying, to a chemical agency, or to mechanical forces.

The chemical idea was first suggested by MÜLLER-THURGAU (18, 19) and strongly supported by MOLISCH (17) and GORKE (8), the theory being that



death resulted from some chemical action on the protoplasm effected by the removal of water from the protoplasm when ice is formed in the intercellular spaces. MOLISCH supposed the injury was caused by a toxic concentration of the cell sap, the increase in concentration being brought about by the dehydration. According to GORKE, the disintegration was thought to be caused by the precipitation of protective substances from the sap at their eutectic points leaving toxic materials to injure the protoplasm.

MOLISCH found that when filaments of *Spirogyra* were exposed to low temperatures, a crust of ice was formed around each of them. He believed that the water from which this ice was formed came from the interior of the cells, dehydrating them until the concentration of certain substances in the cell sap became so great as to injure the protoplasm. He could see no ice formed inside the cells and concluded, therefore, that death was caused entirely by this dehydrating effect. Our results with the *Sedum* cells have indicated that if a thin aqueous film is present around the cells, this layer of water is converted into ice when the freezing point is reached, and the behavior of the protoplast cannot easily be followed. Undoubtedly such a film was present around the algal filaments. This might explain why MOLISCH observed that the cells were dead after they had been thawed, even though he could see no ice inside them. WIEGAND (26) clearly demonstrated the presence of ice within the intercellular spaces of dormant buds, but these buds survived even though the cells adjacent to the ice were so dehydrated that the cell walls were compressed. According to the chemical theory the cells should have been killed by the high concentration of the cell sap. Several investigators have offered rather conclusive evidence that death on freezing is not caused by toxicity resulting from dehydration, and therefore increased concentration, of cell contents.

MAXIMOV (15), ILJIN (9), LEVITT and SCARTH (13, II) and CHANDLER and HILDRETH (6) support the theory that death at low temperatures is the result of mechanical injury either from ice crystals or from strong dehydration, rather than of chemical injury. ILJIN's suggestion that sometimes the killing is caused by the crushing of the protoplasm between the ice formed in the intercellular spaces and that formed in the vacuole is subject to serious criticism. He states that the concentration of the cell sap is less than that of the protoplasm and, therefore, ice must first be formed within the vacuole. LEVITT and SCARTH (13, I) seem to agree with this. They say: "When ice forms inside the cell, its site is the vacuole, not the protoplasm." In a later paper SCARTH and LEVITT (23) reiterate that intracellular ice formation, when it occurs, is confined to the sap vacuole. In their earlier paper they refer to CHAMBERS and HALE (3) as supporting this, but the latter, in describing the freezing in epidermal cells of the bulb scales of the red onion, cite cases and show photographs indicating ice in the cytoplasm external to

the tonoplast or vacuolar membrane when no ice was visible in the vacuole. In their summary CHAMBERS and HALE say: "The ice sometimes broke the outer membrane of the protoplast but the vacuole or tonoplast remains intact, in which case the tonoplast deplasmolyzes on thawing. Ice was not definitely seen to form within the vacuole but the general impression was obtained that it did so form in some cases."

Theoretical considerations indicate that ice formation would occur most readily outside the wall, followed in order by ice formation outside the outer protoplasmic membrane, then within the cytoplasm, and lastly within the vacuole. The osmotic concentration of the vacuole cannot be less but must be equal to or greater than that of the surrounding cytoplasm, for otherwise water would move to the outer, stronger solution. If the vacuolar membrane exerts any pressure whatever on the contained sap—and the surface tension alone of the membrane must be responsible for some pressure on the contents—this sap must be more concentrated than the bathing solution. If the vacuolar sap were less concentrated than that in the cytoplasm and froze at a higher temperature, it would be comparable to having a cell remaining turgid in a hypertonic solution and freezing at a higher temperature than the bathing solution. This situation could not obtain with a membrane permeable to water as the vacuolar membrane must be. Of course, although the vacuolar sap has a higher concentration and would therefore have a lower freezing point than the cytoplasm, the amount of freezing point lowering must be slight and this alone would not be important in preventing freezing. The combination of a membrane which would interfere with ice inoculation, together with this lower freezing point, may be responsible for ice formation in the cytoplasm first. Furthermore, since ice inoculation is likely to take place from the outside, this would favor ice formation in the cytoplasm before that in the vacuole even if they were at the same concentration. SCHANDER and SCHIAFFNIT (24) describe ice formation as a thin layer just inside of the cell wall and between the protoplasm and the wall. The observations here reported, as well as those of SCHANDER and SCHIAFFNIT, and of CHAMBERS and HALE (3), together with the theoretical considerations all indicate that ice formation takes place in the cytoplasm or between the cytoplasm and the wall before it does in the vacuole.

SCARTH and LEVITT (23) state that "Since at equilibrium 'suction tension,' and therefore the freezing point, must be the same in every part of a cell—vacuole, protoplasm, the cell wall—the osmotic value of the cells measured plasmolytically tells us the freezing point of the whole tissue." It is true that the suction tension of the cell as a whole and its wall are in equilibrium so that the freezing point at the surface of the cell is determined by the suction tension of the cell. That is, if cell *a* has zero suction tension, as it would have in distilled water, while cell *b* has a suction tension equivalent

to 0.5 M, ice would form at or in the wall of *a* at the freezing point of distilled water, while at the surface of cell *b* it would form only at or below the temperature necessary to freeze a 0.5 M solution, but in both cases the freezing point of the parts within the outer membrane may be very different and will be independent of the suction tension of the cell. For example, the suction tension of the nucleus, the vacuole, or a plastid will be determined not by the suction tension of the cell but by the osmotic concentration of their own immediate bathing media with which they are in equilibrium. As explained above if the vacuolar or plastid membranes exert any pressure on their contents, their osmotic concentrations must be higher and their freezing points lower than the cytoplasm within which they lie. Different parts may therefore have different freezing points and the osmotic concentration of the vacuole does not give an exact measure of the freezing point of the cytoplasm.

The low resistance to freezing observed by ILJIN (10) when the intercellular spaces of leaves are filled with water, as compared with normal turgid leaves or wilted leaves, presents a problem which would rarely occur under natural conditions. This free water would greatly favor ice formation in the cell. In those experiments in which ILJIN observed a protective action of sucrose or  $\text{CaCl}_2$  when frozen tissues were thawed in the solutions, there is no proof that ice was formed within the cells and possibly in some cases not even in the intercellular spaces. He suggests that in most cases death occurs, not when ice formation takes place in the tissues but when the ice melts. The cells would, of course, be dehydrated by the formation of ice in the intercellular spaces. He suggests that when thawing occurs the walls and protoplast rapidly imbibe water and this rapid dilation or unequal swelling of different parts tears the protoplasm, thus resulting in death. If, on the other hand, this rapid or unequal swelling can be reduced by thawing in strong sugar solutions or those of  $\text{CaCl}_2$  or similar salts of di- or trivalent cations which prevent rapid water absorption, killing is prevented; or the number of cells remaining alive is increased.

Two alternative explanations, however, may be offered. Possibly at the lower freezing temperature all of the cells were killed, but the strong salt or sugar solutions prevented complete deplasmolysis, and the cells therefore merely appeared alive. In the experiments with red cabbage here reported, frozen cells appeared alive if thawed in strong sugar solutions but on transference to dilute solutions or water they were found to be dead, whereas tissues which had not been killed by freezing retained their contents when placed in distilled water or dilute solutions. Another alternative is that, on slow freezing, ice had not formed in the cells but between them. Thawing in water or air may have supplied free water which would be available for ice formation in the cells which were undercooled or were at a temperature

low enough to form ice in the cell when water became available either from the melting intercellular ice or from the applied water or dilute solution. ILJIN gives no evidence that ice ever formed in cells that survived. In our own experiments killing occurred invariably when ice formed in the cells and, so far as was determined, only then.

Evidence from many sources, notably ÅKERMAN (1), ILJIN (11), and CHANDLER and HILDRETH (6), has demonstrated that a high osmotic concentration in cells increases their resistance to freezing. That the presence of solutes lowers the freezing point of the cell is obvious but it is not so obvious why there is often no direct relation between the increase in concentration and increased resistance. The failure to observe a direct relation between osmotic concentration and resistance to freezing is probably chiefly attributable to the fact that differences in rates of chilling, in permeability of the cell membrane to water, or differences in ratio of surface to volume of cell, and differences in amount of undercooling in part due to differences in readiness of ice inoculation across the outer membrane, may largely determine whether ice does or does not form in the cells. For example, a slow lowering of the temperature, high permeability to water, large surface relative to volume, and interference of the membrane to ice inoculation will greatly favor ice formation outside of the cells, thus bringing about a gradual increase in osmotic concentration inside the cell, with perhaps complete failure to form ice within the cells at the temperature to which the cells have been subjected. If the surface membrane interferes with inoculation across it, the fact that the ice outside the cell will have a lower vapor pressure than the undercooled solution inside may further favor outward movement of water, an increased osmotic concentration in the cell, and a lessened likelihood of intracellular ice formation. These several factors may explain the fact that a slight increase in osmotic concentration may be associated with greatly increased frost resistance.

Some investigators hold the opinion that hydrophilic colloids play a more important part than true solutes in increasing cold resistance, and suggest that these colloids by binding water prevent its loss from the cell and thus prevent killing by desiccation. ROSA (22) and NEWTON (20, 21) have consistently found increased bound water in hardened plants and suggest that the increase in bound water is responsible for the increased cold resistance. Many seem to think that the retention of water within the cell by the colloids is in itself significant. It is hard to imagine, however, that such bound water is any more available to the cell than if it were not there at all. The colloidal material itself, on the other hand, may serve as a protective colloid, perhaps preventing precipitation by its peptizing action; or better still, by reducing the free water and increasing the osmotic concentration would thus reduce the likelihood of ice formation.

CHRYSLER (7) found that in kelp stems the total amount of bound water increased as the amount of free water increased. If this holds for most plant materials tending to bind water (and there is a fair amount of evidence that it does), one would expect soaked seeds to contain more bound water than dry seeds, but the higher the water content of the seeds at the time of freezing the less is their resistance. With an increase in free water, on the other hand, even though there is an accompanying increase in bound water, there is an increased probability of ice formation and also a decreased resistance. This strongly points to lack of ice formation, not increased bound water, as responsible for resistance and that ice formation, not desiccation, is responsible for the injury. MEYER (16) actually found more bound water as well as more free water per gram of dry matter in the leaves of *Pinus rigida* in the summer, when the needles were not cold resistant, than in the winter when they were cold resistant.

Our experiments with hardened and non-hardened wheat leaves showed that free water was available for ice formation in the non-hardened leaves at  $-10^{\circ}$  to  $-12^{\circ}$  C. and they were killed at this temperature, but no ice could be observed in the section of hardened leaves until the temperature was lowered to  $-25^{\circ}$  to  $-27^{\circ}$  C. The resistance of dry wheat seed to ice formation and to killing, while those with more water formed ice and were killed, is further evidence that killing is not caused by desiccation or absorption of water after desiccation but to ice formation in the cells.

CHANDLER and HILDRETH (6) coated pollen of peach and *Amaryllis* with castor oil or cotton-seed oil, placed it on ice at  $-15^{\circ}$  to  $-17^{\circ}$  C., and water at  $0^{\circ}$  C. was slowly dropped on the pollen so that it froze as fast as it was added. If the oil was added to the dry pollen as it came from the anthers the freezing caused no injury, but if the pollen was first moistened no protective effect was noted. This is in agreement with our findings with dry and moistened seeds. It would seem that the oil had prevented absorption of water by the dry pollen and therefore no ice formed in the cells; but the moistened pollen had enough free water to allow for internal ice formation, and therefore killing, at the temperature used. The protective effect of the sugar solution is similar to that observed by ÅKERMAN and others, and is probably caused by an osmotic withdrawal of water and thus by prevention of ice formation within the cells.

From the observations here reported and the experiments of other workers the balance of evidence seems strongly in favor of the theory that death of plant tissues at freezing temperatures is brought about by mechanical injury due to ice formation within the cell. Anything that will decrease the amount of free water present will increase resistance to these low temperatures since ice is less likely to form within the cells. We have in no case found any cells to remain alive after ice once formed within them.

SCHANDER and SCHIAFFNIT (24) and ÅKERMÄN (1), however, cite cases in which they observed cells occasionally to remain alive after a small amount of ice was formed within them. By avoiding undercooling and cautiously lowering the temperature SCHANDER and SCHIAFFNIT observed a thin layer of ice to form on the inner side of the cell wall at about  $-5^{\circ}\text{C}$ . If the tissue was then thawed the cells seemed alive as tested by plasmolysis with glycerine. If the temperature was further lowered the layer of ice grew thicker and the cells were killed. ÅKERMÄN found that epidermal cells of red cabbage would withstand a small amount of internal ice formation without killing at  $-5.2^{\circ}\text{C}$ ., but further lowering to  $-7^{\circ}$  or  $-8^{\circ}\text{C}$ . resulted in death. He found that any ice formation in cells of *Rhoeo* resulted in death. These cases of intracellular ice formation without killing were exceptional. They seem to demonstrate that ice can form in cells without killing but it is also conceivable that the ice formed between the wall and the cytoplasm, or that the injury was so slight that only the outer layer of cytoplasm was injured and that the tonoplast or vacuolar membrane was still intact and was responsible for the osmotic responses. CHAMBERS and HALE (3) observed ice formation in and disintegration of the cytoplasm and nucleus without destruction of the tonoplast membrane. It seems then that ice formation within the cytoplasm is almost certain to result in death and perhaps always so. Many claim that ice formation outside of the cell is usually responsible for the killing by freezing because of its dehydrating effect, but although one would expect such dehydration we know of no conclusive evidence that ice formation outside of the cell alone ever causes killing.

The observations of LEVITT and SCARTH (13, II) that cells hardened against freezing are much more permeable to water, strongly support the interpretation here given. As the temperature of the hardened cells is lowered the water would readily move through the surface membrane and form ice outside of the cell and therefore cause no injury. For the non-hardened cells free water would move out much more slowly and ice formation would be more likely to occur within the cell and result in its death. The conditions that reduce the likelihood of ice formation within the cell all seem to favor resistance to freezing. These are low amounts of free water within the cell, high osmotic concentration, high permeability of the membranes to water, small size of cell (high surface relative to volume), slow rate of temperature fall, and a membrane that favors undercooling by preventing inoculation across it.

### Summary

1. A technique is described for direct microscopical observation of the freezing of living cells. When water or sap is present on the outer surface of cells the formation of an ice layer outside of the cells obscures what is

happening in the cells. Freezing in cells without this water film was observed when mounted in paraffin oil.

2. As the temperature of the cells of stamen hairs of *Zebrina pendula* was lowered, streaming became slower and finally ceased. Occasionally Brownian movement was visible for a few seconds after streaming stopped. Then ice formation took place at about  $-7^{\circ}$  C., appearing first in the basal cells and advancing across the field to the tip. Ice crystals were uniformly granular.

3. In the prothallia of *Polypodium aureum* granular crystals were usually produced, but occasionally needle like crystals were formed. Freezing occurred either cell by cell, or a wave of crystallization swept across the field. Insofar as could be determined the order of freezing agrees with the theoretical considerations; ice was formed first in the cytoplasm, then in the vacuole, and lastly in the plastids. The behavior of the nucleus could not be determined.

4. On thawing, the surface membrane was found to have lost its semi-permeable character, allowing droplets of cell sap to collect in the mounting medium; the cytoplasm became granular and disintegrated; the nucleus assumed a glassy appearance.

5. Curves giving data on germination show that seeds of both the wheat varieties, Minhardi and Leap, were more resistant to freezing temperatures when the water content was low. Microscopic freezing tests of sections from the embryos of seeds containing varying amounts of water showed ice formation in seeds with high water and poor germination, and lack of ice in those with low water and good germination.

6. Leaves from plants of Minhardi wheat which had not been hardened showed ice formation at  $-10^{\circ}$  to  $-12^{\circ}$  C., whereas hardened leaves resisted ice formation down to  $-25^{\circ}$  C. Death always resulted if ice was formed in the cells.

7. Sucrose solutions did not protect strips of red cabbage epidermis from the injurious action of ice within the protoplast. If the cells were somewhat plasmolyzed by the solution before freezing began, a lower temperature was required for ice formation.

8. The evidence here presented and the observations of other workers strongly suggest that the death of plant tissues at freezing temperatures is caused by mechanical injury resulting from ice formation within the cells. Anything which will decrease the amount of free water within the cell at the time of freezing is likely to decrease the possibilities of ice formation within the cytoplasm and thus increase the resistance of the cell to freezing temperatures.

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# TEMPERATURE EFFECTS UPON THE GROWTH OF EXCISED ROOT TIPS

GLADYS C. GALLIGAR

(WITH ONE FIGURE)

## Introduction

Little work has been reported on the effect of different temperatures upon the growth of excised root tips in sterile nutrient solutions. WHITE (6) working with root tips of wheat found the optimum temperature for their growth to be between 26° and 27° C. This investigation was undertaken to determine the effect of different temperatures upon the growth behavior of excised root tips of dent corn, cotton, sunflower, and Burpee's Extra Early pea.

## Materials and methods

Uniform lengths of 10 mm. were used throughout all series. Root tips of the varieties just mentioned, when ready for the experiment, were placed in five thermostats, maintained at temperatures of 10, 15, 20, 25 and 30° C. respectively, where they remained for a period of fourteen days. Five individuals for each temperature were used in each of five separate replicate series. Due to losses from contamination, data are reported for only twenty individuals at each temperature. Daily increments of growth were measured until the root tips passed the measurable stage, which occurred as early as the sixth day in sunflower at 20° C.

The procedure used to secure sterile root tips has been outlined in previous papers (1, 2). The nutrient solution was a modification of Pfeffer's formula to which dextrose and peptone were added.<sup>1</sup> Immediately after being made, the solution was measured into the flasks, which were closed with cotton plugs and autoclaved at 15 pounds pressure for 20 minutes.

## Discussion

The results, presented in condensed form in tables I, II, III, IV, and V, demonstrate clearly the irregularity of growth even at uniform temperatures, and prove conclusively that the range in room temperature in previous studies (1, 2) cannot be employed to account for the irregular growth obtained.

<sup>1</sup> Ca(NO <sub>3</sub> ) <sub>2</sub>	2.0 gm.	KCL	0.25 gm.	Dextrose	2.0 per cent.
KH <sub>2</sub> PO <sub>4</sub>	0.5 gm.	MgSO <sub>4</sub>	0.5 gm.	Peptone	0.04 per cent.
KNO <sub>3</sub>	0.5 gm.	FeCl <sub>3</sub>	0.005 gm.	Distilled H <sub>2</sub> O	6000 cc.

TABLE I

SAMPLE SERIES OF FIVE INDIVIDUALS EACH OF SUNFLOWER, COTTON, CORN, AND PEA SHOWING DAILY INCREMENTS IN MILLIMETERS OF LENGTH OF EACH INDIVIDUAL FOR FOURTEEN DAYS AT 10° C.

Sunflower														
1	0	4	1	5	0	5	2	1	2	1	0	0	1	1
2	0	5	2	5	1	3	1	0	1	0	0	1	1	0
3	3	1	1	5	5	0	2	1	2	0	1	1	0	0
4	2	4	4	2	8	2	2	0	0	1	0	1	0	2
5	3	4	1	5	8	0	2	0	2	1	0	1	0	1
Cotton														
1	1	0	0	1	2	0	1	1	1	0	0	2	0	1
2	0	1	0	0	1	1	0	0	0	0	0	0	0	0
3	1	0	0	1	0	0	0	0	0	0	0	0	0	0
4	1	1	0	1	0	0	0	1	0	0	0	0	0	0
5	0	0	1	0	1	0	1	0	0	0	0	1	0	0
Corn														
1	2	2	3	3	3	1	1	2	1	0	0	1	0	0
2	2	4	3	5	2	4	2	4	4	2	2	1	2	3
3	0	4	4	5	2	3	5	2	1	2	1	0	0	0
4	2	1	1	2	4	2	0	3	2	3	2	1	2	2
5	2	3	4	2	1	1	2	2	1	1	0	1	2	3
Burpee's Extra Early pea														
1	2	0	1	1	3	0	7	2	3	3	0	1	4	7
2	1	0	1	2	1	2	3	2	1	2	0	3	3	3
3	1	0	1	1	3	5	2	2	3	3	2	0	3	2
4	1	1	1	1	1	0	0	0	1	1	1	0	1	0
5	1	0	1	1	3	2	2	0	1	3	0	1	3	2

The root tips kept at 10° behaved similarly in some respects and quite dissimilarly in others. In no case was there an indication of laterals in sunflower, cotton, or pea, and only an occasional short lateral in corn. Characteristic papillae like those described previously (1, 2) developed on the sunflower root tips, but no laterals ever pushed through them. These papillae-like elevations were formed by secondary roots pushing outward against the epidermis. The proximal ends of the pea roots frequently became brownish red toward the end of the experiment; the same region of the cotton roots was often slightly blackened; anthocyanin developed to a considerable extent in corn; and sunflower roots retained their natural translucent appearance throughout the experimental period. On the whole, none of the roots were pigmented to any great degree at 10° C. It is of interest to note that an individual root tip of pea attained the greatest length of any root tip grown at 10° C., and that the root tips of pea were exceeded only by the root tips of corn in average total length and in dry weight at the end

of the fourteen-day period. These data indicate that the root tips of pea were more able to maintain growth activities under the given conditions than were those of cotton and sunflower. The increase in diameter of roots of corn and pea grown at 10° C. was markedly greater than that of the same species grown at 20° C., but less than when grown at 15, 25, and 35° C. The thickening was due to enlargement and proliferation of parenchyma cells of the cortex.

At 15° C. the root tips of pea did not branch; cotton and sunflower produced short laterals; and about ten per cent. of the individual root tips of corn produced twenty-five to forty laterals. Sunflower roots developed papillae, through some of which laterals made their way. The development of anthocyanin in corn was equal to that developed at 10° C.; the pea roots became pigmented at their proximal ends; cotton was somewhat more blackened than at 10° C.; and sunflower displayed no pigmentation. Corn root tips surpassed all others in total average length, in average number of

TABLE II

SAMPLE SERIES OF FIVE INDIVIDUALS EACH OF SUNFLOWER, COTTON, CORN, AND PEA SHOWING DAILY INCREMENTS IN MILLIMETERS OF LENGTH OF EACH INDIVIDUAL FOR FOURTEEN DAYS AT 15° C.

	Sunflower													
1	4	2	2	2	2	1	1	0	1	0	1	2	2	1
2	2	2	3	3	2	3	2	3	2	1	2	2	3	2
3	0	2	1	0	1	0	1	0	0	1	1	0	1	1
4	2	2	2	2	2	2	1	3	0	1	2	0	3	2
5	2	1	3	3	2	2	2	2	1	1	2	3	0	1
	Cotton													
1	3	0	0	3	2	0	2	1	1	1	1	1	0	1
2	1	2	1	1	2	0	1	2	2	1	1	0	2	1
3	2	1	1	3	2	2	2	0	1	0	2	2	3	1
4	1	1	2	0	1	2	0	0	2	1	2	2	1	0
5	3	0	2	3	2	2	1	1	2	2	0	2	2	1
	Corn													
1	3	2	3	4	5	2	3	3	2	3	2	1	2	3
2	2	1	2	0	8	2	4	4	5	8	2	1	3	2
3	4	4	5	4	2	2	2	2	2	1	3	2	2	2
4	3	4	3	2	1	7	3	4	6	5	2	2	3	2
5	2	3	4	3	2	4	3	5	3	5	4	2	3	4
	Burpee's Extra Early pea													
1	2	2	2	2	0	0	0	0	1	1	1	0	1	2
2	3	1	1	4	0	1	1	0	0	0	1	2	1	0
3	2	1	2	1	1	1	0	0	0	0	1	1	1	0
4	0	1	1	1	0	0	1	0	0	0	1	0	0	1
5	0	0	1	1	1	1	2	1	0	0	1	1	1	1

TABLE III

SAMPLE SERIES OF FIVE INDIVIDUALS EACH OF SUNFLOWER, COTTON, CORN, AND PEA SHOWING  
DAILY INCREMENTS IN MILLIMETERS OF LENGTH OF EACH INDIVIDUAL  
FOR FOURTEEN DAYS AT 20° C.

Sunflower													
1	11	12	14	8	8	9							
2	5	10	8	7	9	9							
3	5	5	1	1	4	5							
4	4	6	3	4	5	4							
5	5	4	6	3	5	4							
Cotton													
1	4	1	6	7	7	6	5	7					
2	3	1	2	4	3	3	2	2	1	3	1	1	2
3	4	2	5	1	1	1	2	5	4	1	2	1	2
4	0	2	1	2	2	3	4	1	0	2	2	1	1
5	3	2	4	1	2	2	2	3	3	1	2	2	1
Corn													
1	1	1	2	6	6	7	5	5	6	7			
2	1	5	4	4	12	7	5	4	6	4			
3	4	5	4	5	1	1	2	7	5	4			
4	3	4	4	4	6	6	5	4	3	5			
5	4	3	3	5	7	6	4	3	3	5			
Burpee's Extra Early pea													
1	2	3	3	0	2	0	1	0	0	0	1	0	0
2	3	1	1	0	2	0	0	0	0	1	0	0	1
3	3	0	1	5	1	0	1	0	0	0	0	1	0
4	2	1	0	0	2	0	0	0	0	0	0	2	0
5	2	2	1	1	0	0	0	1	0	0	1	0	1

branches, and in dry weight. Pea roots surpassed the roots of sunflower and cotton in dry weight, but surpassed cotton only in total length, and again as at 10° C. produced the longest single root tip.

At 20° C. corn and sunflower showed most branching, cotton produced a few short laterals, and pea none. There were a few individuals among corn and sunflower roots that had no laterals. These sharp individual variations from general tendencies were possibly due to genetic differences. Most of the laterals branched again within the fourteen-day period. Many of the corn roots developed anthocyanin. Cotton showed more blackening than at lower temperatures, many of the roots becoming completely pigmented. In total average length, dry weight, and number of branches corn exceeded all the roots, followed in order by sunflower, cotton, and pea.

At 25° C. corn and sunflower showed less branching than at 20° C., while cotton branched more. There was no pigmentation in corn, pea, and sunflower, but cotton became completely brown or black at the end of the

experimental period. The root tips of cotton excelled in average length of root and in average number of branches, while corn root tips were first in dry weight. This indicates that the protoplasm of the cotton roots was stimulated to excessive growth activity at this particular temperature with a resultant greater use of stored reserves in the older portion of the root.

At 35° C. only a few individuals of corn produced lateral roots, which were never more than five in number and always short and thickened. Pea and cotton became pigmented, but there was no coloration in sunflower or corn. At this temperature all the corn roots became much thickened due to hypertrophy of the cortex, which in some instances split away from the central cylinder, a condition found in earlier work (1, 2). Cotton and pea elongated on the average during the two week period about half again the length of the original fragment of root, sunflower about twice the original length, and corn three times the original length. In dry weight sunflower,

TABLE IV

SAMPLE SERIES OF FIVE INDIVIDUALS EACH OF SUNFLOWER, COTTON, CORN, AND PEA SHOWING DAILY INCREMENTS IN MILLIMETERS OF LENGTH FOR EACH INDIVIDUAL FOR FOURTEEN DAYS AT 25° C.

	Sunflower													
1	6	5	2	3	2	1	1	0	1	2	2	2	1	2
2	3	5	2	3	6	6	5	4	3	2	2	5	3	2
3	3	4	5	6	4	4	4	0	1	3	4	3	3	4
4	6	5	4	3	3	4	3	0	2	1	2	3	4	2
5	4	3	4	2	2	4	3	3	0	3	1	2	0	2
	Cotton													
1	3	4	4	4	10	13	12							
2	1	0	4	5	8	8	9	7						
3	3	2	7	8	6	6	8							
4	4	2	3	4	0	1	1	5	6	5	6	4	5	
5	4	5	5	4	11	14	9							
	Corn													
1	2	7	7	0	6	11	2	2	3	3				
2	1	4	4	7	6	8	3	3	2	2	4	3	2	
3	5	4	4	10	2	5	7	6	3					
4	3	5	5	8	4	5	10	4	2					
5	2	9	8	4	7	12	3	2	4					
	Burpee's Extra Early pea													
1	1	1	1	1	0	1	0	0	0	0	0	0	1	0
2	2	1	1	0	1	0	0	0	1	0	0	1	0	0
3	2	2	1	2	1	5	0	0	0	0	0	0	0	0
4	2	1	1	1	1	0	0	1	0	0	1	0	0	0
5	1	2	1	1	1	1	0	0	0	1	0	0	0	1

TABLE V

SAMPLE SERIES OF FIVE INDIVIDUALS EACH OF SUNFLOWER, COTTON, CORN, AND PEA SHOWING DAILY INCREMENTS IN MILLIMETERS OF LENGTH FOR EACH INDIVIDUAL FOR A PERIOD OF FOURTEEN DAYS AT 35° C.

	Sunflower													
1	1	1	0	0	1	0	1	0	0	0	1	0	0	0
2	1	1	1	0	3	0	1	1	0	1	1	0	1	0
3	2	0	1	0	3	0	0	0	0	0	0	0	0	0
4	2	0	1	0	0	1	0	0	1	0	0	1	0	1
5	1	0	1	1	0	2	0	0	1	0	0	0	0	1
	Cotton													
1	3	1	1	0	1	0	0	0	0	1	1	0	0	0
2	4	0	1	0	1	2	0	0	0	0	0	0	1	3
3	4	1	0	1	0	0	0	0	0	0	0	1	0	0
4	2	0	1	1	1	0	0	0	0	1	0	0	1	0
5	3	1	1	1	0	0	0	0	1	0	0	0	1	1
	Corn													
1	1	2	1	2	1	1	1	1	0	0	0	1	0	0
2	0	2	4	1	1	2	1	0	0	0	1	2	1	1
3	2	2	3	1	0	1	3	0	1	1	0	0	1	1
4	3	3	3	2	3	1	0	0	1	1	0	0	1	1
5	2	2	1	2	3	0	1	0	0	0	1	1	1	1
	Burpee's Extra Early pea													
1	0	1	1	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	1	0	1	1	0	0	0	1	0	0	0	0	0	0
5	1	0	1	0	1	1	0	0	0	0	0	0	0	0

cotton, and pea were approximately equal, while corn attained a dry weight of nine to ten times that of the other three.

The behavior of the root tips of pea was unusually interesting. Average total length and average dry weight indicate that these roots perform best at a temperature of 10° C. At 15° C. they also performed better than at the higher temperatures, 20° and 25° C., but the dry weight at 15° C. was 50 per cent. greater than it was at 20 and 25° C. Since the root tips used in this experiment grew better under the given conditions at 10° C., it cannot be said in general that 10° C. is the optimal temperature for the growth of pea roots. It is the optimal temperature for the prescribed conditions for a fourteen day period. It is possible that the lower temperature inhibited the absorption of some constituent in the medium, which, when absorbed at higher temperatures exercised an inhibitory effect upon the growth activities of the root tips. On the other hand the better growth may have been caused by an internal response to the lower temperature. There



TABLE VI

SUMMARY OF DATA ON 600 ROOT TIPS CUT AT ORIGINAL LENGTHS OF 10 MILLIMETERS AND ALLOWED TO GROW 14 DAYS AT DIFFERENT TEMPERATURES IN THE BASIC SOLUTION

TEMP- PERA- TURE ° C.	SPECIES	AVERAGE DAILY INCREMENT OF LENGTH OF 20 ROOT TIPS FOR 1ST 10 DAYS										AV. TOTAL LENGTH OF 14 DAYS	RANGE IN LENGTHS OF INDIVIDUAL AT END OF 14 DAYS	AV. MUL- TIPICA- TION OF THE ORIGINAL LENGTH IN 14 DAYS	AVER- AGE NO. OF LATERAL ROOTS	RANGE IN NO. OF LATERAL ROOTS AMONG INDI- VIDUALS	AV. DRY WT. PER 10 ROOTS
		1	2	3	4	5	6	7	8	9	10						
10	Corn	mm. 1.53	mm. 2.71	mm. 2.73	mm. 3.77	mm. 3.75	mm. 2.50	mm. 2.03	mm. 2.58	mm. 2.24	mm. 2.00	mm. 39.	mm. 20-45	3.9	0.3	0-4	gm. 0.125
	Pea	mm. 1.21	mm. 0.23	mm. 1.00	mm. 1.24	mm. 2.00	mm. 2.10	mm. 3.00	mm. 1.00	mm. 2.56	mm. 2.00	mm. 36.	mm. 17-65	3.6	0.0	0	0.035
	Sun- flower	mm. 1.29	mm. 3.51	mm. 1.73	mm. 4.20	mm. 3.50	mm. 2.51	mm. 1.26	mm. 0.20	mm. 0.78	mm. 0.20	mm. 34.	mm. 16-49	3.4	0.0	0	0.027
	Cotton	mm. 0.70	mm. 0.20	mm. 0.00	mm. 0.53	mm. 0.59	mm. 0.34	mm. 0.00	mm. 0.23	mm. 0.20	mm. 0.27	mm. 14.	mm. 10-23	1.4	0.0	0	0.022
15	Corn	mm. 3.00	mm. 2.70	mm. 2.67	mm. 2.50	mm. 4.55	mm. 1.75	mm. 4.00	mm. 3.00	mm. 3.10	mm. 5.20	mm. 41.	mm. 24-70	4.1	5.0	0-40	0.114
	Pea	mm. 1.70	mm. 1.52	mm. 1.23	mm. 2.01	mm. 0.30	mm. 0.20	mm. 0.51	mm. 0.00	mm. 0.00	mm. 0.20	mm. 29.	mm. 16-76	2.9	0.0	0	0.032
	Sun- flower	mm. 2.04	mm. 2.30	mm. 2.12	mm. 1.70	mm. 1.75	mm. 1.56	mm. 1.23	mm. 1.50	mm. 0.70	mm. 0.20	mm. 32.	mm. 15-60	3.2	2.0	0-10	0.025
	Cotton	mm. 1.70	mm. 1.05	mm. 1.11	mm. 1.72	mm. 1.67	mm. 1.10	mm. 1.69	mm. 0.47	mm. 0.73	mm. 0.20	mm. 13.	mm. 10-30	1.3	0.62	0-8	0.030
20	Corn	mm. 2.21	mm. 3.54	mm. 3.70	mm. 5.21	mm. 4.73	mm. 7.02	mm. 4.70	mm. 5.20	mm. 4.08	mm. 4.58	mm. 109.	mm. 53-152	10.9	41.0	0-75	0.200
	Sun- flower	mm. 6.24	mm. 4.71	mm. 6.57	mm. 5.00	mm. 6.52	mm. 3.22	mm. 3.74	mm. 2.79	mm. 3.07	mm. 3.24	mm. 57.	mm. 23-110	5.7	11.0	0-43	0.034
	Cotton	mm. 2.77	mm. 1.54	mm. 3.20	mm. 3.60	mm. 3.41	mm. 0.00	mm. 0.50	mm. 0.03	mm. 0.00	mm. 0.22	mm. 43.	mm. 21-71	4.3	9.0	3-14	0.028
	Pea	mm. 2.52	mm. 1.26	mm. 1.24	mm. 0.20	mm. 2.74	mm. 0.00	mm. 0.50	mm. 0.03	mm. 0.00	mm. 0.22	mm. 30.	mm. 24-95	3.0	0.0	0	0.023
25	Corn	mm. 7.53	mm. 5.00	mm. 5.50	mm. 4.50	mm. 4.57	mm. 6.73	mm. 1.75	mm. 1.23	mm. 1.26	mm. 1.25	mm. 57.	mm. 32-118	5.7	14.0	0-66	0.090
	Cotton	mm. 2.70	mm. 2.00	mm. 4.59	mm. 5.20	mm. 7.54	mm. 7.00	mm. 7.57	mm. 1.23	mm. 1.26	mm. 1.25	mm. 98.	mm. 30-136	9.8	30.0	14-45	0.033
	Sun- flower	mm. 5.10	mm. 4.72	mm. 3.26	mm. 3.74	mm. 3.67	mm. 3.58	mm. 3.90	mm. 1.00	mm. 1.56	mm. 2.30	mm. 44.	mm. 17-67	4.4	4.0	0-15	0.028
	Pea	mm. 1.78	mm. 1.20	mm. 1.07	mm. 1.06	mm. 0.73	mm. 1.58	mm. 0.00	mm. 0.20	mm. 0.02	mm. 0.02	mm. 29.	mm. 16-76	2.9	0.0	0	0.021
35	Corn	mm. 1.51	mm. 2.22	mm. 3.06	mm. 1.21	mm. 1.03	mm. 1.26	mm. 1.05	mm. 0.07	mm. 1.00	mm. 0.52	mm. 30.	mm. 23-37	3.0	0.46	0-5	0.076
	Sun- flower	mm. 1.56	mm. 0.50	mm. 0.73	mm. 0.00	mm. 1.70	mm. 0.21	mm. 0.53	mm. 0.30	mm. 0.00	mm. 0.56	mm. 19.	mm. 12-35	1.9	0.0	0	0.008
	Cotton	mm. 2.21	mm. 1.01	mm. 0.50	mm. 0.53	mm. 0.76	mm. 0.54	mm. 0.00	mm. 0.00	mm. 0.00	mm. 0.56	mm. 15.	mm. 13-20	1.5	0.0	0	0.006
	Pea	mm. 0.00	mm. 0.21	mm. 0.20	mm. 0.00	mm. 0.00	mm. 0.00	mm. 0.00	mm. 0.00	mm. 0.00	mm. 0.00	mm. 13.	mm. 11-15	1.3	0.0	0	0.007

TABLE VII

## SUMMARY OF THE DATA ORGANIZED TO SHOW THE BEHAVIOR OF EACH SPECIES

SPECIES	TEM- PERA- TURE	AVERAGE DAILY INCREMENT OF LENGTH OF 20 ROOT TIPS FOR 1ST 10 DAYS										AV. TOTAL LENGTH AT END OF 14 DAYS	RANGE IN LENGTHS OF INDIVIDUAL AT END OF 14 DAYS	AV. MUL- TIPLICA- TION OF THE ORIGINAL LENGTH IN 14 DAYS	AVER- AGE NO. OF LATERAL ROOTS	RANGE IN NO. OF LATERAL ROOTS AMONG INDI- VIDUALS	AV. DRY WT. PER 10 ROOTS
		1	2	3	4	5	6	7	8	9	10						
		mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.	mm.						
Corn	° C.											mm.	mm.				gms.
	10	1.53	2.71	2.73	2.77	3.73	2.50	2.03	2.58	2.24	2.00	39.	20-45	3.9	0.3	0-4	0.125
	15	3.00	2.70	2.67	2.50	4.55	1.73	4.00	3.10	3.10	5.20	41.	24-70	4.1	5.0	0-40	0.114
	20	2.31	3.54	2.70	5.21	4.73	7.02	4.70	5.20	4.08	4.58	109.	53-152	10.9	41.0	0-75	0.200
	25	7.53	5.00	5.50	4.50	4.57	6.73	1.75	1.23	1.26	1.25	57.	32-118	5.7	14.0	0-66	0.090
35	1.51	2.22	3.06	1.21	1.03	1.26	1.05	0.07	1.00	0.52	30.	23-37	3.0	0.46	0-5	0.078	
Sun- flower	10	1.29	3.51	1.73	4.20	3.50	2.51	1.26	0.20	0.78	0.20	34.	16-49	3.4	0.0	0	0.027
	15	2.04	2.30	2.12	1.70	1.75	1.56	1.23	1.50	0.70	0.20	32.	15-60	3.2	2.0	0-10	0.025
	20	6.24	4.71	6.57	5.00	6.52	3.67	3.58	3.90	1.56	2.30	57.	23-110	5.7	11.0	0-43	0.034
	25	5.10	4.72	3.26	3.74	3.62	3.58	3.90	1.00	1.56	2.30	44.	17-67	4.4	4.0	0-15	0.028
	35	1.56	0.50	0.73	0.00	1.70	0.21	0.53	0.30	0.00	0.56	19.	12-35	1.9	0.0	0	0.008
Cotton	10	0.70	0.20	0.00	0.53	0.59	0.54	0.00	0.23	0.20	0.27	14.	10-23	1.4	0.0	0	0.022
	15	1.70	1.05	1.11	1.72	1.67	1.10	1.69	0.47	0.73	0.20	13.	10-30	1.3	0.62	0-8	0.030
	20	2.77	1.54	3.20	3.60	3.41	3.22	3.74	2.79	3.07	5.24	43.	21-71	4.3	9.0	3-14	0.028
	25	2.70	2.00	4.59	5.20	7.54	7.00	7.57	0.00	0.00	0.56	98.	30-136	9.8	30.0	14-45	0.033
	35	2.21	1.01	0.50	0.53	0.76	0.54	0.00	0.00	0.00	0.56	15.	13-20	1.5	0.0	0	0.006
Pea	10	1.21	0.23	1.00	1.24	2.00	2.10	3.00	1.00	2.56	2.00	36.	17-65	3.6	0.0	0	0.035
	15	1.70	1.52	1.23	2.01	0.30	0.20	0.51	0.00	0.00	0.20	29.	16-76	2.9	0.0	0	0.032
	20	2.52	1.26	1.24	0.20	2.74	0.00	0.50	0.03	0.00	0.22	30.	24-95	3.0	0.0	0	0.023
	25	1.78	1.20	1.07	1.06	0.73	1.58	0.00	0.20	0.20	0.02	29.	16-76	2.9	0.0	0	0.021
	35	0.00	0.21	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	13.	11-15	1.3	0.0	0	0.007

was little growth at 35° C. The roots exhibited marked increase in diameter at 10 and 25° C. owing to enlargement of the cortical cells. No lateral roots developed at any temperature used.

Root tips of corn gave their best performance at 20° C., which is 12° C. lower than the optimum temperature found by LEHENBAUER (3) for the growth of young shoots of corn. LEHENBAUER pointed out that the optimum temperature for growth under the conditions of his experiments shifted somewhat as the time factor was varied.

Sunflower also behaved best at 20° C. At 10° and 25° C. no lateral roots appeared within the fourteen day period, but the characteristic papillae, which frequently appeared in sunflower, and from which laterals later protruded, occurred on many individuals at both temperatures, but they occurred in greater numbers on those at 10° C. than on those at 35° C. In no case did a moniliform root develop, which was probably due to the fact that the time period used was too short for such a phenomenon to occur. In many individuals at 35° C. the proximal ends of the root tips became unduly thickened owing to a proliferation of cortical parenchyma and to excessive enlargement of cells of the same tissue. It is of interest to note that in final average total length and in average dry weight the root tips at 10° C. slightly exceeded those at 15° C. This observation may possibly have some correlation with the behavior of colloidal substances in the protoplasm, which are known to manifest peculiar reactions in the neighborhood of 15° C., which differ from those occurring at higher and lower temperatures.

Cotton showed optimal average total length and optimal average dry weight at 25° C. Compared with cotton roots grown at room temperature as long as growth would continue, the 25° cultures averaged more than 50

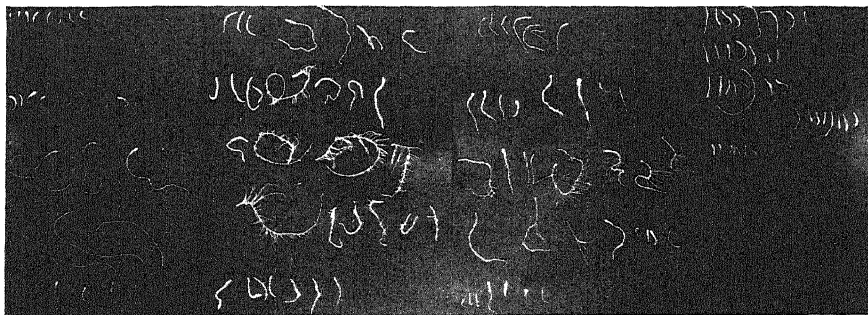


FIG. 1. Root tips maintained at constant temperatures for 14 days. Reading from left to right are four groups of root tips: cotton, corn, sunflower, and pea respectively. In each group the tips in the top row were maintained at 10° C., in the second row at 15° C., in the third row at 20° C., in the fourth row at 25° C., and in the bottom row at 35° C.

per cent. longer, and approximately 25 per cent. greater in dry weight. There was scarcely any growth in length at 10° and 35° C. An examination of the dry weight figures for these temperatures indicate that the root tips at 35° C. drew heavily upon the reserves in the older part of the root in order to remain alive.

Figure 1 presents a few samples of each species of root tip from each temperature used, and shows at a glance general trends in the behavior of all the root tips used at the various temperatures.

### Summary

1. The optimal temperature for excised pea roots was 10° C., for sunflower and corn 20° C., and for cotton 25° C.
2. Few or no lateral roots developed at temperatures of 10° and 35° C.
3. Temperatures of 25° and 35° C. inhibited the formation of anthocyanin in corn.
4. Temperatures of 10° and 15° C. retarded pigmentation in cotton.
5. Marked distension in diameter occurred in roots of pea at 10° and 25° C. and in corn and sunflower roots at 35° C.
6. 35° C. was definitely beyond the range of optimal temperature for growth of root tips of corn, pea, sunflower, and cotton.

JAMES MILLIKIN UNIVERSITY  
DECATUR, ILLINOIS

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# CARBOHYDRATES OF BEAN PLANTS AFTER TREATMENT WITH INDOLE-3-ACETIC ACID

TAYLOR R. ALEXANDER

(WITH SEVEN FIGURES)

## Introduction

During recent years, a number of histological and morphological studies of the tumor-growth responses of bean plants to indole-3-acetic acid have been published (1, 4, 6). One investigation of etiolated bean plants indicated that indole-3-acetic acid (heteroauxin) affects the translocation of carbohydrate and nitrogenous material (7). The general stimulating effect of indole-3-acetic acid on the synthesis of solid matter by bean plants is known (6). WENT and THIMANN (10) state that in the *Avena* coleoptile there are deposited on the cell wall large amounts of cellulose, pectin, and hemicelluloses, but that there is no direct relationship between the amount of these substances and the amount of auxin applied. They state further that the action of auxin in these processes is indirect.

Although one would expect the rates of respiration to differ between indoleacetic-acid treated and control plants, reports have not confirmed this assumption (7).

The present investigation concerns some of the carbohydrate changes which are related to the structural and weight differences previously observed in plants treated after decapitation.

## Investigation

### MATERIALS AND TREATMENT

The plant used was the Dwarf Red Kidney bean, *Phaseolus vulgaris*. The seeds were selected for uniformity in size and in appearance. Two crops, of approximately 1,000 plants each, were grown, crop 1, Feb. 20 to Mar. 10, and crop 2, Feb. 22 to Mar. 12. The plants were grown in quartz sand in 5-inch clay pots. A more uniform population was obtained, and the shedding of the seed coats was facilitated, when the seeds were planted with the raphe facing downward, the seed inclined at an angle of 45°. After planting, the pots were watered with warm tap water. The temperature of the greenhouse fluctuated from 58° to 90°F. during the course of the experiment, but averaged about 75°F. The relative humidity varied from 30 to 80 per cent., but was generally below 50 per cent. Crop 1 germinated during very cloudy weather, while crop 2 came up under sunshine of high light intensity. After Feb. 27, the sun was bright except for three days.

Six days after planting, the plants had emerged through the surface of the sand. The plants were then culled, leaving a very uniform crop, and the pots were watered with warm nutrient solution (8). The nutrient treatment was repeated every fourth day, the sand being kept moist with tap water between the additions of nutrients.

Ten days after planting, the plants were about nine inches high, and the second internode was one to two inches long. The crops were divided into three equal groups, of approximately 300 plants each—initial controls, final controls, and treated plants. The second internode was cut off 2 cm. above the point of divergence of the primary leaves. The initial control group was then harvested. The cut surface of the final control group was covered with 3 to 4 mm.<sup>3</sup> of lanolin. The third group was treated with the same amount of lanolin in which indole-3-acetic acid had been thoroughly mixed. The two per cent. paste was made by melting 5 gm. of anhydrous lanolin, and vigorously stirring into it 100 mg. of indole-3-acetic acid (Merck). Following treatment, the treated and untreated plants were placed in alternate rows, so that an equal number of both were exposed to nearly the same environmental factors. In all lots axillary shoots were removed as soon as they appeared as small buds.

Seven days after treatment, the final controls and treated plants were harvested and divided into the following samples: upper stem end, lower stem end, primary leaves, stem, and roots (see fig. 1). The roots were freed of sand by dipping them momentarily into a saturated salt solution and then washing thoroughly with distilled water. The initial controls had been harvested in a like manner.

#### PHYSICAL MEASUREMENTS

Fresh weights of all samples were determined by use of a torsion balance. The tissue was then placed in a well-ventilated drying oven, and dried to constant weight at 80°C. The oven-dried samples were placed in dry Mason jars, sealed, and allowed to cool. In this way moisture adsorption was minimized, and later, dry weights were determined. A torsion balance, sensitive to 0.01 gm., was used for the large, and an analytical balance for the small samples.

Before the stem ends could be ground, the lanolin had to be removed. This was accomplished by a one-minute extraction with petrol ether. Alpha-naphthol tests on the ether after extraction showed a very faint carbohydrate test. The ends were redried, and a new dry weight was taken from which to calculate the data.

#### CHEMICAL METHODS

The small samples were ground by hand with mortar and pestle until the powder would pass an 80-mesh screen. The large samples were finely

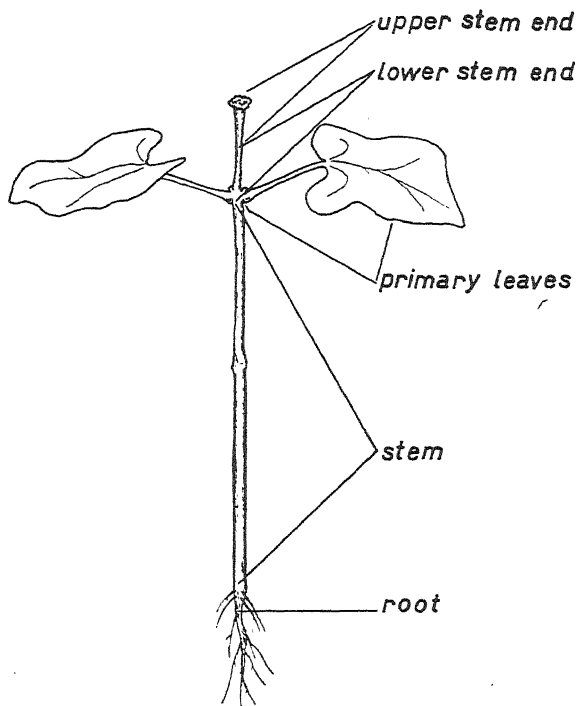


FIG. 1. Division of plant into samples.

ground in a coffee mill. Then a 5- to 10-gram portion was ground to 80-mesh fineness by the use of a ball mill. All samples were redried at 80°C., and placed in a desiccator.

The analyses were run on duplicate 1-gm. samples for crop 1 and single samples for crop 2. These were placed in weighed filter papers, and extracted with hot 75 per cent. alcohol in Soxhlet extractors on a sand bath. Each extraction was checked for completeness by the alpha-naphthol test. At the end of the first hour the alcohol in the receiving flasks was replaced by a fresh quantity to prevent overheating of the sugars, as suggested by LOOMIS and SHULL (5).

The alcohol-soluble sugar extract was prepared and analyzed by the ceric sulphate method of HASSID (2, 3), using Setopaline C as the indicator. Sucrose was also determined by this method, after using invertase for inversion.

After the sugar extraction, the net residue was weighed. The sample was then ground to 100-mesh fineness, redried, and an aliquot was weighed out for the determination of polysaccharides. Starch and dextrin, water-soluble acid-hydrolyzable, and insoluble acid-hydrolyzable substances were hydrolyzed and calculated according to the procedure of LOOMIS and SHULL.

Saliva was used for the starch and dextrin hydrolyses, and 1 + 20 HCl was used for the other two hydrolyses. The above-mentioned carbohydrate fractions were determined as reducing sugars on the cleared and neutralized extracts by the ceric sulphate method.

The solution containing the acid-hydrolyzable substances showed considerable color, and determinations made upon duplicate colored samples did not check with sufficient accuracy. The samples were, therefore, decolorized by treating a 25-cc. aliquot of the neutralized solution with 0.3 gm.

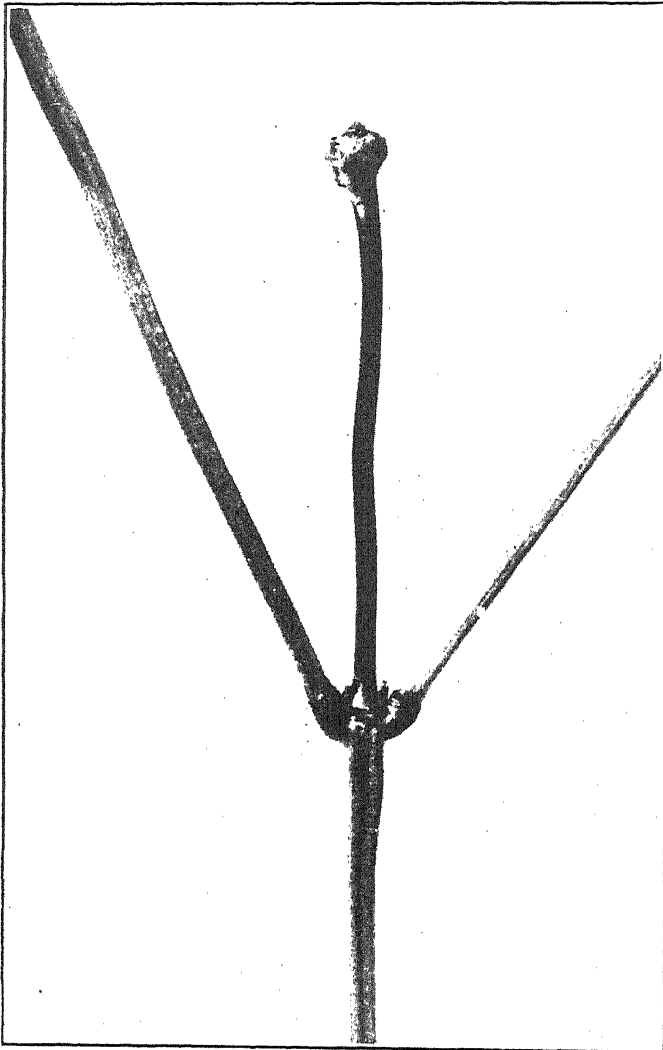


FIG. 2. Treated stem end at time of harvest.



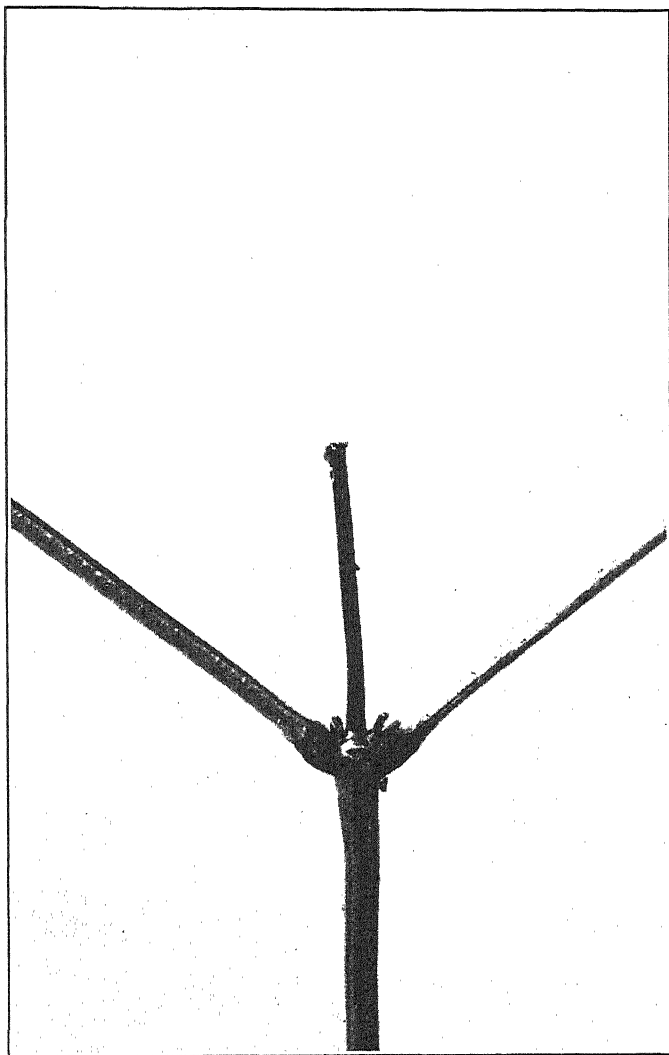


FIG. 3. Control stem end at time of harvest.

of charcoal for 15 minutes, after which it was filtered with suction. The sample was then brought up to a 100-cc. volume, and aliquots of this cleared solution were used for the titrations. With this modification, duplicate determinations checked very closely.

## Results and discussion

### MORPHOLOGICAL OBSERVATIONS

The response of the plants to the heteroauxin treatment was identical to that reported previously (4, 6) (see figs. 2 and 3). Tumors and roots

were produced, the former shown in figure 2. The final control plants produced a large number of small calluses at the cut surface.

WEIGHT DIFFERENCES

Examination of table I shows that the upper stem end responded to the indoleacetic acid treatment by an increase of approximately five times as much dry weight as the final control. The entire stem end, both upper and lower portions, shows about a 300 per cent. gain (see figs. 4 and 5). The treated stem ends of crop 2 gained more than those of crop 1.

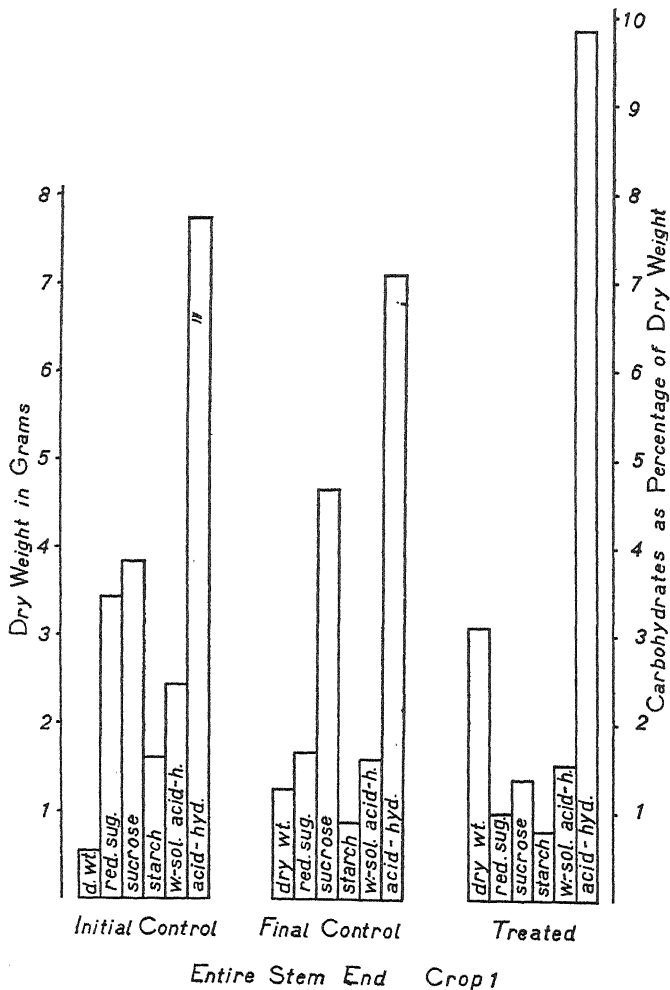


FIG. 4. Comparison of dry weight and chemical composition (data based on grams per 100 plants).

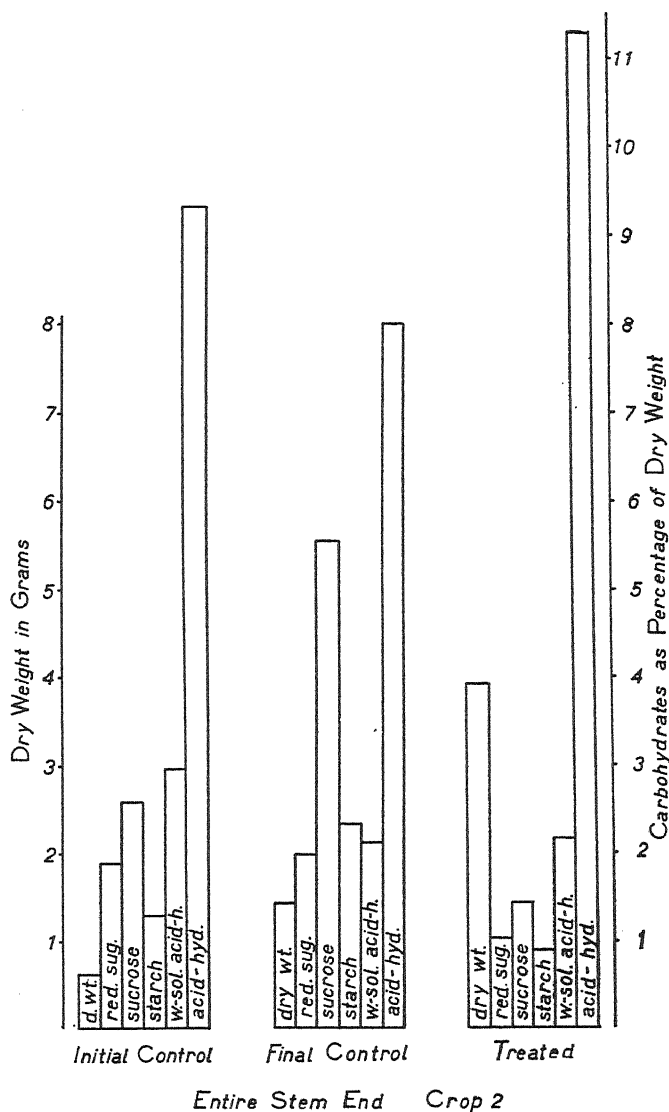


FIG. 5. Comparison of dry weight and chemical composition (data based on grams per 100 plants).

With regard to the entire plant, the total weight of the final controls is heavier in both crops (figs. 6 and 7). There is a greater difference between the dry weight of the final control and treated plants in crop 2 than in crop 1. The primary leaves account for most of the differences in total dry weight, but it is not a matter of increase in leaf area following heteroauxin application (6).

TABLE I

DRY WEIGHTS (FIGURES REPRESENT GRAMS OF DRY WEIGHT PER 100 PLANTS)

REGION	INITIAL CONTROL	FINAL CONTROL	TREATED
Crop 1			
Upper stem end .....	0.16	0.40	1.87
Lower stem end .....	0.39	0.86	1.44
Primary leaves .....	20.22	40.63	35.92
Stem .....	10.93	19.00	18.17
Roots .....	7.21	12.00	11.61
Total .....	38.91	72.86	69.01
Crop 2			
Upper stem end .....	0.17	0.42	2.28
Lower stem end .....	0.44	1.00	1.64
Primary leaves .....	18.17	45.83	39.18
Stem .....	8.64	19.22	17.68
Roots .....	6.92	13.43	12.79
Total .....	34.34	79.90	73.57

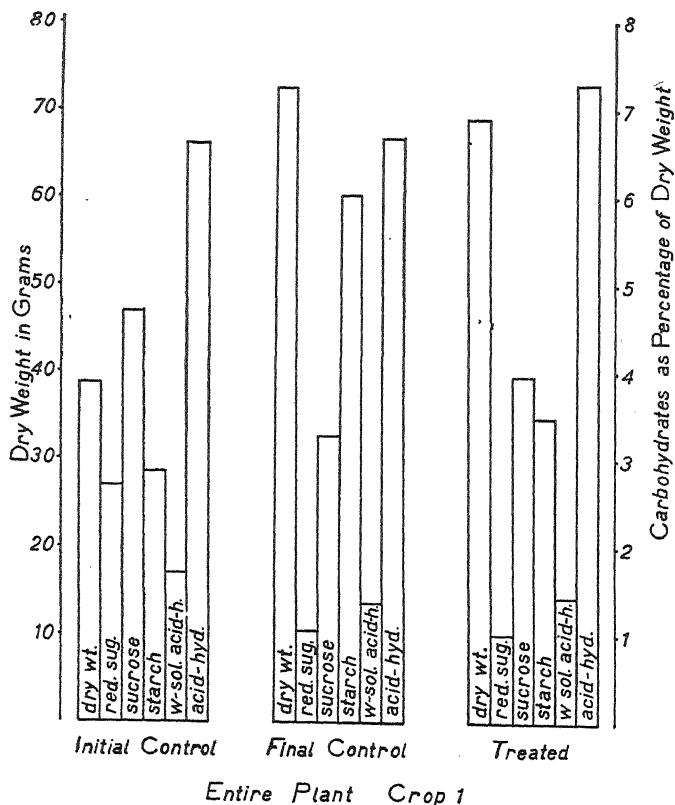


Fig. 6. Comparison of dry weight and chemical composition (data based on grams per 100 plants).

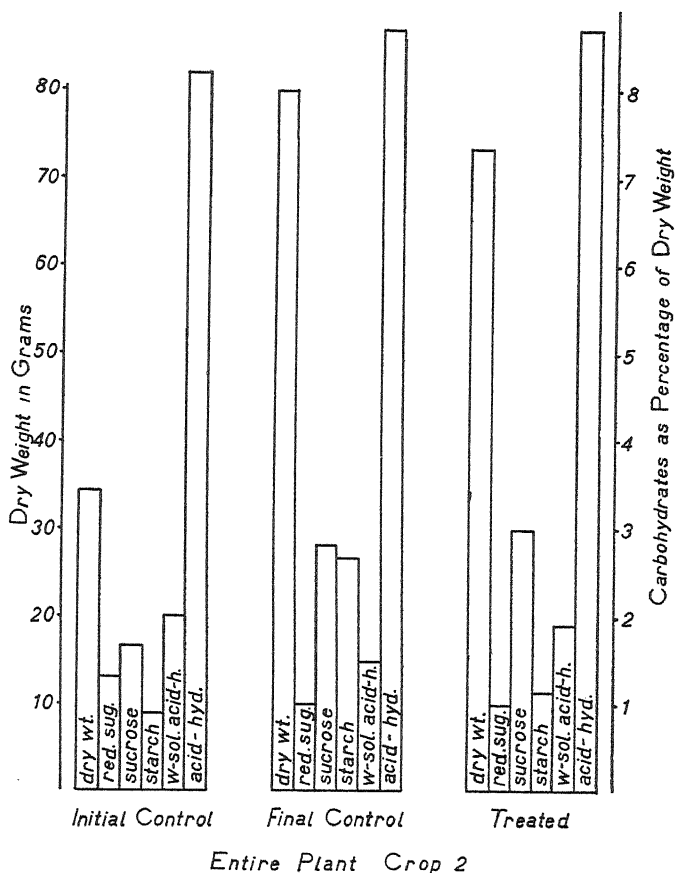


FIG. 7. Comparison of dry weight and chemical composition (data based on grams per 100 plants).

The increase in weight seems to indicate several things. First, small changes in the environment cause quantitative differences in the response to indoleacetic acid treatment. As indicated before, crop 1 was grown under lower light intensity than crop 2, especially during the early stages of development; and, therefore, crop 1 was etiolated in appearance as compared to crop 2. The effect is evidenced in the amount of dry matter synthesized both in the entire plant and in the stem end. Second, the decrease in dry weight of the primary leaves, stems, and roots of the treated plants indicates a movement of material to the region where heteroauxin had been applied (6). Third, the loss in weight of these parts is greater than the gain made by the stem ends, so it seems highly probable that a part of the translocated substances was respired in the very active tissues of the tumor. Fourth, crops 1 and 2 have a 5.3 and 7.9 per cent. difference in weight,

respectively, in favor of the final control plants; the stem ends of crop 1 are the lighter. In other words, the stem end of crop 2 gained 17.8 per cent. more than those of crop 1, and, at the same time, the primary leaves, stems, and roots of the treated plants of crop 2 lost 33 per cent. more than those of crop 1. The assumption that the treated plants were capable of and did synthesize at least as much dry matter as the final controls, and that there really was a loss in the treated plants, is based on the work of MITCHELL and HAMNER (6). They found that plants treated in a similar manner with two per cent. indoleacetic acid paste had gained, at the end of six days, in dry weight over the controls, if the axillary shoots were not removed. At the same time, a large tumor was formed. The additional leaf area evidently supplied enough material for the tumor growth and in addition the heteroauxin stimulated the photosynthetic process. Points three and four suggest to the writer that, under the conditions of the experiment, respiration is accelerated in the treated stem end to furnish energy for the excessive growth and translocation processes.

#### CHEMICAL COMPOSITION

Figures 4, 5, 6 and 7, and table II give the chemical composition of the various plant parts. The upper stem end shows, in general, smaller percentages of the more complex fractions than the lower stem end. This indicates that there is another substance or substances present in large amount. These substances might be nitrogenous in nature, but belong most likely to the structural polysaccharides. The chemical nature of the entire stem end of the treated plants of both crops is very similar. The main difference is a greater percentage of water-soluble acid-hydrolyzable and insoluble acid-hydrolyzable material in crop 2. This may be associated with differences in light intensity. The final control plants follow the same trend with respect to the last two groups of compounds mentioned; in addition they are high in sucrose.

The higher percentage of carbohydrates in the final controls of crop 2 suggests that photosynthetic activity was favored by the more intense light. As compared to the treated stem ends, the metabolically active carbohydrates are higher instead of the insoluble acid-hydrolyzable materials. The difference in the reducing sugar and sucrose content of the initial controls of the two crops can be partially explained by the fact that crop 1 was harvested late in the afternoon, while crop 2 was harvested late at night when one would expect condensation to have taken place and respiratory losses to have occurred. Glucose is such a variable quantity that no great stress should be laid on its variation for interpretation of studies of this kind.

The entire plants of both crops have a chemical composition that is com-

TABLE II

CHEMICAL COMPOSITION (DATA AS PERCENTAGE OF DRY WEIGHT PER 100 PLANTS)

REGION	REDUCING SUGARS			SUCROSE			STARCH			WATER-SOLUBLE ACID-HYDROLYZABLE			ACID-HYDROLYZABLE		
	INITIAL CON-TROL	FINAL CON-TROL	TREATED	INITIAL CON-TROL	FINAL CON-TROL	TREATED	INITIAL CON-TROL	FINAL CON-TROL	TREATED	INITIAL CON-TROL	FINAL CON-TROL	TREATED	INITIAL CON-TROL	FINAL CON-TROL	TREATED
Crop 1															
stem end	6.18	1.60	1.07	3.88	5.50	1.29	1.69	0.80	0.87	3.06	1.57	1.51	6.50	5.80	8.90
stem end	2.28	1.76	0.88	3.82	4.29	1.45	1.62	0.92	0.75	2.16	1.62	1.56	8.24	7.70	11.15
✓ leaves ..	2.64	0.82	0.96	5.47	2.85	3.42	4.79	8.22	5.54	1.94	1.31	1.44	4.76	5.08	4.99
.....	3.20	1.58	1.29	4.98	5.33	3.58	0.69	4.90	1.56	1.30	1.22	1.30	8.16	8.02	9.23
.....	1.59	0.75	0.60	2.14	1.27	0.70	0.76	0.75	0.76	1.66	1.70	1.59	9.38	10.03	10.50
plant .....	2.69	1.03	1.00	4.71	3.25	3.95	2.83	6.04	3.46	1.71	1.36	1.43	6.61	6.68	7.28
Crop 2															
stem end	2.82	2.86	1.01	1.11	7.61	1.01	1.12	3.48	0.78	3.62	2.69	2.15	8.36	6.36	10.18
stem end	1.57	1.62	1.04	2.46	4.72	2.01	1.36	1.84	1.01	2.73	1.88	2.17	9.65	8.70	13.17
✓ leaves ..	1.16	1.05	0.99	1.69	3.00	4.12	0.51	1.33	1.02	2.38	1.38	1.86	6.33	7.85	6.87
.....	1.74	1.15	1.13	2.16	3.45	2.56	1.80	7.04	1.63	1.62	1.47	1.95	10.18	9.17	9.95
.....	1.16	0.67	0.70	0.96	0.88	0.66	0.74	0.89	0.78	1.87	1.81	1.94	10.60	11.56	12.00
plant .....	1.32	1.00	0.98	1.68	2.80	3.00	0.90	2.64	1.12	2.02	1.49	1.91	8.20	8.76	8.74

parable. In the case of the more intense light conditions (crop 2), sucrose and starch of the primary leaves of the final controls tend to be transformed into insoluble acid-hydrolyzable substances. Leaves of the treated plants are higher in sucrose and insoluble acid-hydrolyzable substances instead of high in starch.

In the case of higher light conditions (crop 2), starch and insoluble acid-hydrolyzable substances of the final control stems are higher, while sucrose is lower. The stems of the treated plants are higher in insoluble acid-hydrolyzable and water-soluble acid-hydrolyzable substances in place of the other fractions.

The roots of the crops showed no significant differences in chemical composition, except that in each case the acid-hydrolyzable fraction was higher in the treated plants as compared with the final controls.

If the stimulating effect of the indoleacetic acid increases the photosynthetic activity of the leaves, then despite the increased translocation of sugar to the stem end, there is an accumulation of sucrose in the leaves of the treated plants as compared with the controls. The fact that the stimulus is absent from the control plants probably accounts for the conversion of soluble carbohydrates to starch and insoluble acid-hydrolyzable substances. But in the stem of the treated plants all the carbohydrates are low except the insoluble acid-hydrolyzable materials. Thus, in the treated plants, it seems evident that there is translocation of carbohydrates to the stem end.

The water-soluble acid-hydrolyzable substances are thought to consist mainly of pectins and gums (5). The insoluble acid-hydrolyzable material is considered to be composed of pectic substances (9), insoluble pentosans, protopectins, galactans, polymers of uronic acid, and a portion of the cellulose (5). Assuming compounds of the above types to be present, indole-3-acetic acid apparently produces a condition that causes soluble carbohydrates to move to the region of application and there to be synthesized into the higher polysaccharides. The previously-mentioned anatomical studies reported great meristematic activity, with lignin and secondary thickenings being laid down in the pith, phloem, endodermis, cambium, and other tissues. This chemical study agrees with those findings.

A greater percentage of the dry weight is accounted for in the final controls than in the treated plants. This suggests further that cellulose is a likely component of the remainder of the residual dry weight in the case of the treated plants.

Energy for growth is supplied through respiratory processes. Soluble sugars are low at the points of stimulated growth, and respiration as well as condensation may account for this fact. Examination of figures 4 and 5 shows that in crop 2, for which seemingly more favorable growth conditions



prevailed and greater growth occurred, the soluble respirable sugars are lower than in crop 1.

### Summary

1. The stem ends of bean plants were removed and treated with two per cent. indole-3-acetic acid in lanolin. Tumors and roots appeared at the point of treatment.

2. Reducing sugars, sucrose, starch and dextrin, water-soluble acid-hydrolyzable substances, and insoluble acid-hydrolyzable materials were determined.

3. Dry weight was greater in the final control plants than in the treated plants.

4. There was a translocation of carbohydrates toward the point of treatment. The final controls differed from the treated in having a greater percentage of starch.

5. Stimulation by indole-3-acetic acid brought about conditions causing simple carbohydrates to be condensed to complex polysaccharides at the point of application.

6. Losses in dry weight of the treated plants indicated respiratory increases. Reduction of soluble carbohydrates in the treated stem ends was caused by increases in acid-hydrolyzable substances, and probably also by respiration incident to stimulated metabolic activity of the tumor.

The writer wishes to acknowledge his appreciation to Dr. J. W. MITCHELL for suggesting this problem, and to Professors C. A. SHULL and S. V. EATON for encouragement and suggestions.

THE UNIVERSITY OF CHICAGO  
CHICAGO, ILLINOIS

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THE MIRSKY-PAULING THEORY OF THE STRUCTURE OF  
NATIVE, DENATURED, AND COAGULATED PROTEINS,  
AND SOME THEORETICAL ASPECTS OF THE EVOLU-  
TION OF OXYGEN FROM THE IRRADIATED  
GREEN PLANT<sup>1</sup>

O. L. INMAN

This paper calls attention to some possible relationships which may exist between the sensitization and denaturation of proteins and one phase of the mechanism of photosynthesis.

MIRSKY and PAULING (5) point out that the most significant change that occurs in denaturation of a native protein is the loss of certain highly specific properties. The specific property here considered in the case of the process of photosynthesis is the possible denaturation of a protein which brings about the inhibition of the evolution of oxygen from the green plant when irradiated.

It has been shown by INMAN (3) that coagulation of the triturate of leaf tissue by pH, heat, calcium salts, dehydration, surface action, alcohol, etc., inhibits the evolution of oxygen. With pH held below 4.1 or above 8.1 for an hour at about 20° C., there was little indication of reversibility. The action of the combination of heat and dehydration is most interesting. If a whole leaf of *Trifolium repens* is dried in an oven at 30° C. for 48 hours, it may be powdered or used whole and allowed to absorb tap water, after which it will evolve oxygen upon irradiation. If, on the other hand, it is heated 48 hours at 45° C., it will not upon being permitted to absorb tap water evolve oxygen with irradiation. If the leaf, dried slowly at 30° C., is heated to 60° C. for a short time while dry it will still evolve oxygen. When leaves are triturated while fresh and dried at 30° C. for 48 hours, they will no longer evolve oxygen. A few plants such as *Nostoc* will stand extended drying at room temperature and still evolve oxygen upon adding water and visible radiation.

Freezing and thawing 57 times with carbon dioxide did not inactivate the leaf triturate of *Trifolium repens*. Immersion of the meal in liquid air for 30 minutes failed to inactivate it. In fact, the lower the temperature the slower the inhibition of the power to evolve oxygen. The lower alcohols are strong inhibiting agents. Studies of rates of inhibition and effects of surface as influenced by a variety of reagents indicate that the release of oxygen by the plant when irradiated is a chemical reaction, probably influenced by at least one enzyme. MIRSKY points out that denaturation of a protein is a

<sup>1</sup> Contribution from the C. F. Kettering Foundation for the study of chlorophyll and photosynthesis.

definite chemical reaction and not a vague disintegration of the protein, since a structural change in the molecule takes place. The action of crude or crystalline trypsin as an inhibitor for the evolution of  $O_2$ , caused either by the coagulation effect or by proteolytic action, seems to point to the denaturation of some rather specific native protein or protein group in the leaf triturate. This specificity is noteworthy owing to the fact that pepsin shows no inactivation. No recovery has been noted after inactivation by the use of trypsin. Boiled trypsin has no inhibiting action.

The triturated meal, upon standing in air or on being heated, becomes inactivated. The rate of inactivation is accelerated by a rise in temperature. This seems to indicate a chemical reaction, but does not specifically indicate whether a protein is being denatured. The hydrogen ion concentration inactivation curve has the same form as an enzyme hydrogen ion concentration curve. This indicates an enzyme reaction and the enzyme may be protein in nature. Also, the fact that leaves may be heated to a higher temperature when dry than when moist before inactivation occurs, points to a fact that fits in with the finding that some proteins behave as if there is coagulation in the case of freezing or dehydration, but no denaturation, or the molecules do not change in structure. There is, of course, the possibility of reversible denaturation in such a case.

The fact that shaking with fine glass beads inhibits the evolution of oxygen fits with the inactivation of some proteins by surface phenomena.

The proof of the existence of a "Blackman reaction" as well as a photochemical phase in the reactions of carbon fixation by the green plant is not out of line with the idea that a protein may participate in the mechanism as a part of the Blackman or the photochemical phase.

On the one hand, if the chlorophyll pigment as we know it in the pure form is associated with a protein in chemical combination, either the same protein exists in most green plants, or there is a specific active group common to many proteins, or there are a number of proteins to which the chlorophyll may be combined, or there are a number of chlorophylls in green plants. If, on the other hand, proteins merely act as carriers without sufficient bonding strength to enable one to extract the prosthetic group and the proteins together, or if the proteins merely give surface to the chlorophyll pigment, then coagulation with denaturation of the proteins could just as well serve as an inhibiting factor in the release of oxygen. The same may be true if the carbon dioxide, which is the most probable source of the free oxygen, is bound chemically with or absorbed on a protein which would be denatured by various treatments. While convincing experimental evidence is not at hand, it seems that chlorophyll is most probably to be considered as a kind of "prosthetic group" which when in the green plant is combined with an associated carrier. The work by KUHN and RUDY (4) in which a catalytic

cally active flavoprotein was formed is most suggestive and from the biological standpoint provides a method of attack which offers much promise. The work of STERN and SALOMON (8) on oververdin suggests other possible applications to chlorophyll studies. FRENCH (2) considers a pigmented cell extract obtained from *Streptococcus varians* by high frequency sound waves to be a chromoprotein. There is certainly some reason to conceive of combining (chemically or by adsorption) extracted chlorophyll, which is photosensitive, with a carrier which can be sensitized and through this arrangement of radiation, prosthetic and carrier group account for the absorption and reduction of  $\text{CO}_2$ .

A similar system such as is postulated for the visual purple mechanism is not so fantastic even though speculative. Native visual purple  $\xrightleftharpoons[\text{dark}]{\text{light}}$  denatured visual purple (visual yellow)  $\rightleftharpoons$  denatured protein retinene. If there is such a protein sensitization by some substance similar to retinene (in this case, perhaps chlorophyll or carotenoids), the protein or protein complex probably has a higher energy of activation than that of the visual purple mechanism but a lower energy of activation than most proteins.

The most serious difficulties apparent here are the lack of the true chemical structure of chlorophyll, especially as found in the cell, and the ignorance of the cellular metabolic reactions certainly necessary before the final reactions of the mechanism can take place. It is quite probable that studies of rates of reaction as measured by the evolution of  $\text{O}_2$  or absorption of  $\text{CO}_2$  are only the final steps in a long series of chain reactions. That the photosynthetic mechanism is a part of some of the basic physical and chemical reactions within the living cell is hard to refute with the evidence available.

While much work has been done which has led a number of workers to conclude that the enzyme catalase, a protohematin protein-complex, plays a part in the release of oxygen from some organic peroxide, this particular part of the mechanism seems to lack sufficient proof and may be too simple. EMERSON and GREEN (1) have recently reported work which failed to lend support to such a concept. From the work of MOLISCH (6) and INMAN (3), there is much more conclusive evidence that there is an enzyme (probably a native protein-complex) participating in the mechanism of photosynthesis but whether this has anything to do with catalase or an organic peroxide is still not clear. Some of the data on the evolution of oxygen, especially with dehydration at  $30^\circ \text{C}$ . fit in with MIRSKY's and PAULING's conception of coagulation and denaturation.

The probability of protein sensitization playing a part in some phase of the photosynthetic reactions is not to be dismissed without much further study. If some organic molecular complex somewhat similar to the visual

purple mechanism is involved along with the chlorophyll this would render the task of the reduction of carbon dioxide *in vitro* even more precarious until some agent can be found which will combine chemically with carbon dioxide or absorb it in such a manner that it becomes activated by visible light (see SPOEHR, 7). The same fact might be true for the water molecule. There seems no good reason to assume that visible radiation directly activates either carbon dioxide or water since they do not absorb to any extent in the visible region of the spectrum. However, it is certainly possible that water or carbon dioxide or a combination of the two, when associated with chlorophyll which absorbs visible radiation, might become activated and enter into chemical and photochemical reactions one of which could be the release of oxygen.

ANTIOCH COLLEGE  
YELLOW SPRINGS, OHIO

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## BRIEF PAPERS

### HERITABLE VARIATIONS IN CHLOROPHYLL

J. C. IRELAND

(WITH ONE PLATE)

A spectrographic record of chlorophyll extracts is offered to replace the uncertain terms used to describe the degrees of greenness of leaves of grain sorghums in making inheritance studies. Chlorophyll deficiencies are described as albino, virescent, golden, mottled, striped, and with other words implying heritable characteristics (2). It has been found that some varieties of kafir after several generations of inbreeding have a percentage of virescent seedlings. Later developments of the plants show that many of them have an increase of concentration of chlorophyll over the normal plant and that very definite bands appear in spectrographic records. Continued sampling throughout the entire growing period during the last three seasons has revealed some interesting variations in the different strains.

#### Methods

From June to October, each Monday at 9 A. M., collections were made from ten head-to-row plantings. One-gram samples were weighed and ground in coarse sand. The small amounts of leaf material were used to secure uniformity during the early growing period, because seedlings were obtained at a very early stage. After grinding, 20 cc. of 95 per cent. alcohol was added to extract the chlorophyll, and the mixture was filtered after 4 hours. Ordinary glass test tubes were used as containers, but the tubes were marked and the samples were filtered into their respective tubes each week. Photronic cell readings (3) of the solution were made to determine the density and to make possible a comparison with GUTHRIE'S (1) standard solution.

A grating spectrograph was used, with a no. 1 photoflood bulb mounted in a microscope lamp stand as the source of light. The photoflood lamp provides a fairly uniform source of white light. Dufaycolor film was used to record the color, and it was processed immediately after the exposures were completed, following the directions provided by the manufacturer of the film. Exposures of the film were made for thirty seconds for each sample. The first exposure was that of distilled water, followed by GUTHRIE'S solution, then the ten chlorophyll samples. This routine was followed each week during the seasons.

A Zeiss hand sugar refractometer was used to determine the sugar concentrations after the nodes appeared and sugar became sufficiently concentrated to make readings. The juice expressed upon the plate of the refrac-

tometer from the fresh pulp represents a comparatively accurate estimate of the sucrose in the sap.

### Results

Plate IV indicates the results of 1936 and 1937 spectrographic records. Table I lists the various selections of sorghums used in the determinations of the seasons.

TABLE I  
SORGHUM VARIETIES  
CHLOROPHYLL AND SUGAR CONTENT  
1936-1937

PLATE I SPECTRO- GRAPHIC NO.	VARIETY OF HYBRID	1936		1937	
		CHLORO- PHYLL PHO- TRONIC	SUGAR PER CENT.	CHLORO- PHYLL PHO- TRONIC	SUGAR PER CENT.
H <sub>2</sub> O	Distilled water .....	00.0	00.0	00.0	00.0
Ck	Guthrie's solution .....	12.8	00.0	10.5	00.0
1 and 1'	Blackhull X Red Darso .....	13.3	12.2	10.6	9.9
2 and 2'	Reed X Milo .....	12.7	11.3	10.4	11.3
3 and 3'	Blackhull X Japonica .....	14.6	12.2	11.1	10.2
4 and 4'	Red Darso X Blackhull .....	14.1	11.1	10.6	10.5
5 and 5'	Pearl .....	15.9	11.3	11.2	11.5
6 and 6'	Red Kafir X Darso .....	14.8	11.2	10.5	9.9
7 and 7'	Red Kafir X Darso .....	14.0	11.9	10.6	9.1
8 and 8'	Blackhull X Japonica .....	12.9	12.3	10.1	13.1
9 and 9'	Blackhull .....	13.3	12.1	10.4	9.6
10 and 10'	Black Amber X Durra .....	13.8	12.9	10.7	7.5

It may be observed that the 1936 concentrations are generally higher than those of 1937, as indicated by the photronic cell readings. The spectrographic records of rows 2 and 2', 9 and 9', and 10 and 10' do not seem to be determined by the intensities of chlorophyll. Rows 5 and 5' indicate a

### EXPLANATION OF PLATE IV SPECTROGRAPHIC COMPARISON OF CHLOROPHYLL EXTRACTS

H <sub>2</sub> O .....	Distilled water in test tube.
Check .....	GUTHRIE'S solution.
1 and 1' .....	Blackhull X Red Darso.
2 and 2' .....	Reed X Milo.
3 and 3' .....	Blackhull X Japonica.
4 and 4' .....	Red Darso X Blackhull.
5 and 5' .....	Pearl.
6 and 6' .....	Red Kafir X Darso.
7 and 7' .....	Red Kafir X Darso.
8 and 8' .....	Blackhull X Japonica.
9 and 9' .....	Blackhull.
10 and 10' .....	Black Amber X Durra.







considerably higher concentration and lack the yellow and red extensions in the spectrographic record. Since these bands are duplicated in successive generations, regardless of concentrations, it may be assumed that they are due to heritable characters.

It may be observed that the chlorophyll and sugar values for 1937 are lower than those of 1936. The chlorophyll readings with the same photronic cell may be lower because of some deterioration of the cell, but the sugar differences are probably caused by variations in the supply of moisture.

### Summary

The spectrographic variations in the chlorophyll extracts of grain sorghums are continuous throughout a season and in two generations. The concentration of chlorophyll and of sugars does not correlate with the widths of spectrographic bands.

OKLAHOMA AGRICULTURAL EXPERIMENT STATION  
STILLWATER, OKLAHOMA

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### NICOTINIC ACID AND THE GROWTH OF ISOLATED PEA EMBRYOS<sup>1</sup>

JAMES BONNER

In earlier publications (2, 3) it has been shown that a number of different chemical substances function as "growth factors" for the isolated pea embryo. These accessory growth factors or their precursors are normally stored in the cotyledons whence they are mobilized by the seedling plant after germination of the seed. For extensive growth *in vitro* of the "cotyledonized" embryo, however, these growth factors as well as the ordinary foods must be supplied in the nutrient medium. By the use of a technique for the *in vitro* cultivation of isolated pea embryos in the dark, it has thus been shown that vitamin B<sub>1</sub>, ascorbic acid, pantothenic acid, and theelin, all function as accessory growth factors for the seedling plant (2, 3). Evidence will be presented here which indicates that nicotinic acid, known to be a

<sup>1</sup> Report of work carried out with the aid of the Works Progress Administration, Official Project no. 465-03-3-342, Work Project no. N-9199.

component of the vitamin B complex and to be indispensable to the growth of certain microorganisms (5), is also a growth factor for the pea seedling.

Peas of the variety Perfection were sterilized in 0.1 per cent.  $\text{HgCl}_2$  and were then soaked in sterile water for 6 hours. At the end of this time the embryos were cut from the cotyledons and were transferred to 50-cc. Erlenmeyer flasks containing 15 cc. of the pea nutrient medium described elsewhere (1, 2) which contains, in addition to inorganic salts, only 4 per cent. sucrose and 1 per cent. agar. All of the operations were, of course, carried out under sterile conditions and the few cultures which subsequently showed infection were discarded. To the basic nutrient medium was added the desired amount of accessory growth factor, in this case nicotinic acid or vitamin  $\text{B}_1$ , or both. The cultures were kept in a controlled dark room at  $24^\circ \text{C}$ . and the shoot length of each plant was recorded weekly for 4 weeks.

Table I gives the result of two parallel experiments which were closely comparable and which have been combined in this table.

TABLE I  
EFFECT OF NICOTINIC ACID AND OF VITAMIN  $\text{B}_1$  ON THE SHOOT GROWTH OF  
ISOLATED PEA EMBRYOS

ADDED GROWTH FACTOR	NUMBER OF EMBRYOS GROWN	SHOOT LENGTH AFTER			
		1 WEEK	2 WEEKS	3 WEEKS	4 WEEKS
None (control) .....	40	<i>mm.</i> 15	<i>mm.</i> 22	<i>mm.</i> 26	<i>mm.</i> 28
Nicotinic acid 0.01 mg./flask .....	40	17	25	32	37
Vitamin $\text{B}_1$ , 0.01 mg./flask .....	40	22	32	40	49
Nicotinic acid + vitamin $\text{B}_1$ , each 0.01 mg./flask .....	40	23	34	50	68

The control embryos to which no accessory growth factor is added are able nevertheless to grow somewhat under these conditions. This is undoubtedly to be attributed in part to the growth factors which the embryo contains as it is isolated from the cotyledons, and in part to a limited capacity of the embryo to synthesize some growth factors from the constituents of the basic medium (3). If 0.01 mg. of nicotinic acid per culture flask is added to the medium, the growth of the shoot is somewhat accelerated, and after 4 weeks the plants supplied with nicotinic acid are one third taller than the controls. Vitamin  $\text{B}_1$  at the same concentration in the medium exerts a considerably larger effect upon the growth of the shoot, and the further addition of nicotinic acid, as shown in the last line of table I, further enhances this effect.

It is now well established that vitamin B<sub>1</sub> in its rôle as a plant growth factor influences primarily the root (1, 2), and the effect of this substance upon the growth of the shoot is probably then the result of its influence on the root system. It is of interest therefore to determine what influence nicotinic acid exerts on the growth of the root. This was investigated by means of experiments in which nicotinic acid was added to the nutrient medium in which isolated pea roots were grown *in vitro*.

Tips were cut from the roots of seedling peas and were grown for one week *in vitro* in order to deplete them of their initial supply of vitamin B<sub>1</sub>. At the end of the week, fresh tips 1 cm. long were cut from these roots and transferred to fresh medium with added growth factors as shown in table II.

TABLE II  
EFFECT OF NICOTINIC ACID AND OF VITAMIN B<sub>1</sub> ON THE GROWTH OF ISOLATED  
PEA ROOTS

ADDED GROWTH FACTOR	NUMBER OF ROOTS GROWN	TOTAL LENGTH AFTER 1 WEEK
		<i>mm.</i>
None (control) .....	40	21 ± 0.5
Nicotinic acid		
0.01 mg./15 cc. ....	40	21 ± 0.6
Vitamin B <sub>1</sub>		
0.01 mg./15 cc. ....	40	48 ± 1.1
Nicotinic acid		
+ vitamin B <sub>1</sub> ,		
each 0.01 mg./15 cc. ....	40	56 ± 1.0

It may be seen from table II that nicotinic acid alone exerts no effect on the growth of the isolated pea root. It does, however, increase somewhat the response of the root to vitamin B<sub>1</sub>. It may be, therefore, that the effects of nicotinic acid upon the growth of the shoot, as shown in table I, are primarily due to effects of this substance upon the root itself.

Nicotinic acid and/or closely related derivatives are known to occur widely in natural products: in leaves, seeds, yeast, etc. (4). It might be suspected *a priori* that this general distribution indicates a function of nicotinic acid in higher plants. From the evidence presented in this paper it may be tentatively concluded that this is indeed the case.

WILLIAM G. KERCKHOFF LABORATORIES OF THE BIOLOGICAL SCIENCES  
CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

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## EFFECTS OF FLUE DUST ON THE GROWTH OF SUNFLOWER AND GOLDEN WAX BEAN

BARNEY BARNETT COHEN

Investigations concerning the influence of flue dust on plant growth have been few and in most cases reported by foreign investigators. In 1915 the *Journal of the Society of Chemical Industry* (5) reports an analysis of flue dust for possible injurious substances and concludes that none were found. VÜRTHEN (11) in analyzing samples of dust from the Kaliasch Iron Works thought that the average percentage of chlorides present was sufficient to reduce the fertilizing value of flue dust. VOELCKER (9) suggested to purchasers that guarantees be demanded not only for the potassium present but for the total *soluble* potassium. He also reports (10) in experiments with wheat plants that not only the percentage of Fe present was to be considered, but also whether the Fe was present in ferrous or ferric state. VLASYAK (8) studied the effects of Fe-Mn flue dust and found that dusts with not more than 4 per cent. alkalies are suitable fertilizers for beets.

In the experiment reported here, flue dust from the Wisconsin Steel Mills, Chicago, was used in varying proportions in the soil. Sunflowers and Golden Wax beans were grown in these soils which contained varying proportions of flue dust and observations and records were made of growth behavior. The analysis of the flue dust, supplied by the Wisconsin Steel Mills, is as follows:

Fe .....	50.34	per cent.
C .....	11.53	“ “
Si .....	10.83	“ “
CaO .....	3.20	“ “
Al <sub>2</sub> O <sub>3</sub> .....	2.76	“ “
Mg .....	0.67	“ “
MgO .....	0.41	“ “
P .....	0.246	“ “

## Procedures

The sunflowers and Golden Wax beans were grown in glazed pots, six being used for each test. One pot, with soil only, was used as a control. The second contained a mixture of one thousand parts of soil to one part of dust; the third, ten parts of soil to one part of dust; the fourth, one part of soil to one part of dust; the fifth, one part of soil to three parts of dust; and the last flue dust alone. The above proportions of dust and soil were thoroughly mixed before being placed in the pots. The plants were watered at two day intervals with tap water, and, observations were recorded from day to day.

Dry and green weights of the sunflower (table I), and number and weights of the fruits of the bean (table II) were recorded. Typical results have been selected as a basis for comparison.

## Data and results

TABLE I

DRY AND GREEN WEIGHTS OF SUNFLOWER

SOIL USED		SOIL ONLY	1000:1	10:1	1:1	1:3	DUST ONLY
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Dry weight .....	Top .....	23.00	31.00	14.9	12.00	5.00	0.84
	Root .....	2.44	3.26	1.02	.83	.351	0.091
	Total .....	25.44	34.26	15.92	12.83	5.351	0.931
Green weight .....	Top .....	129.0	180.2	124.0	112.3	34.0	6.100
	Root .....	10.2	21.0	7.3	5.7	3.1	.930
	Total .....	139.2	201.2	131.3	118.0	37.1	7.03

TABLE II

YIELD OF GOLDEN WAX BEEN FRUITS

	SOIL ONLY	1000:1	10:1	1:1	1:3	DUST ONLY
Number of fruits .....	24	23	21	18	10	None
Weight, grams .....	36.15	36	20.3	21.1	11.1	.....

Results obtained with Golden Wax bean indicate that in excess of 10 per cent. flue dust, anomalous growth effects were noticeable, and became more pronounced with increasing concentrations. Chlorosis of the lower leaves, stunting, delayed flowering, and reduced size and number of fruits were some of the observed results of disturbed metabolism. The growth of the Golden Wax bean in complete flue dust was very meager.

With the sunflower, results of a different nature were obtained. In the mixture of one part dust to one-thousand parts of soil, which is comparable

to additions of fertilizers, increased yields were obtained. As in the Golden Wax bean, concentrations in excess of 10 per cent. showed decreased growth and chlorosis.

### Discussion

The induced chlorosis may be accounted for by two factors: first, the excess of Fe present and, second, the alkalinity of the soil. MARSH and SHIVE (6) believe that the concentration of soluble Fe above the optimum may result in a condition in which the plant is unable to use the Fe present and so suffers from deficiency even in the presence of extremely high Fe concentrations.

Of course, the possibility of deposition of insoluble Fe in stem and root tissues might cause failure of translocation of Fe within the plant. This seems improbable, however, since microchemical tests (7) have shown Fe to be present in upper and lower parts of the bean stem.

The high alkalinity of the flue dust is probably an important factor in inducing iron chlorosis. The results of 4 tests showed pH values of 7.9, 7.9, 8.0, and 7.9. Experiments conducted by JONES (3) and JONES and SHIVE (4) indicate that the decrease of pH from 4.8 to 4.2 increased the availability of Fe when supplied in the form of ferric phosphate.

HOPKINS and WANN (1, 2) have observed also that in *Chlorella* Fe was unavailable at high pH values.

The increased growth of the sunflower in the second pot indicates that this plant is able to utilize the abundance of essential elements provided in the flue dust and at the same time tolerates the high pH value resulting from its application in quantity.

### Conclusions

1. Flue dust, in concentrations in the soil greater than 10 per cent., has a decided effect in reducing the yield of fruits of the Golden Wax bean.

2. In concentrations in the soil ranging near 0.1 per cent. it has a decidedly favorable effect on the growth of the sunflower.

3. In concentration in the soil above 10 per cent., it has a depressing effect upon the growth and yield of the sunflower.

4. Sunflower and Golden Wax bean grown in complete flue dust show meager growth, the effect being more pronounced in the case of the bean.

The writer wishes to express his appreciation to Dr. C. A. SHULL for suggestions in connection with this problem.

THE UNIVERSITY OF CHICAGO  
CHICAGO, ILLINOIS

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## EFFECT OF SUN AND SHADE ON PIGMENT DEVELOPMENT

WILLIAM A. BECK

In a preliminary experiment which preceded a study of seasonal variations of pigment production in field plants, the writer obtained interesting results regarding the effect of sun and shade on the production of pigments in plants.

The work was done in the first week of June, 1936. It was a sunny season and the plants were vigorous. Plantain (*Plantago major* L.) was chosen because specimens could be found in sunny and shady places not far removed from each other. The sun plants were taken near the top of a hill where the slope was slight, and the shade plants were taken from the woods on the same hill where the slope was about the same. Direct sunlight never struck the shade plants, but diffuse light was plentiful throughout the day. The plants in both places had not been disturbed or mutilated from the time of early Spring. The four youngest leaves were chosen for tests in both cases so that the leaves were comparable with respect to the age factor.

It was previously reported by JÖNSSON, LUBIMENKO, STÅLFELT, ROSÉ,

LUNDERGÅRDH, and URSPRUNG that more chlorophyll develops in plants that are shaded than in those that grow in sunny places. Nothing is stated in the literature, so far as the writer's knowledge goes, about the relative amounts of xanthophyll and carotene which develop in direct sun light and in the shade. In the experiments reported here, simultaneous determinations of all three pigments were made for the plants growing in direct sunlight and in the shade.

The comparison of the amount of pigment yielded by plants growing in the two places was made on the basis of leaf area, wet weight, and dry pulp weight. The results obtained by the area method were almost identical with those obtained with the method employing the weight of the dry pulp. Both were considerably different from the results obtained with the wet weight method because the plants growing in direct sunlight contained considerably more water than did the shade plants.

The wet weight per cm.<sup>2</sup> of leaf area was 18.1 mg. for the shade plants and 22.8 mg. for the sun plants. The weight of the dry pulp for shade plants and sun plants was the same, *i.e.*, 3.0 mg. per cm.<sup>2</sup> of leaf area.

The sun plants contained 0.025 mg. of chlorophyll per cm.<sup>2</sup> of leaf area, and the shade plants 0.035 mg. The shade plants contained 40 per cent. more chlorophyll. The sun plants contained 0.0044 mg. of xanthophyll per cm.<sup>2</sup> of leaf area, and the shade plants only 0.0038 mg. The shade plants contained 13.6 per cent. less xanthophyll than the sun plants. The sun plants contained 0.00081 mg. of carotene per cm.<sup>2</sup> of leaf area and the shade plants 0.000828 mg. The shade plants contained slightly more carotene than the sun plants.

The results of the experiment show that direct sunlight favors the storage of water in the plantain, which reminds one of the water storage by succulent plants. The development of chlorophyll is considerably favored by shaded conditions; the same conditions slightly favor the development of carotene. The development of xanthophyll is slightly favored by sunny conditions.

INSTITUTUM DIVI THOMAE  
CINCINNATI, OHIO

TEACHING METHODS<sup>1</sup> IN PLANT PHYSIOLOGY

CHARLES J. LYON

At the annual meeting of the American Society of Plant Physiologists held at Indianapolis in December, 1937, the programs closed with a symposium on teaching methods in plant physiology. The chairman, Dr. CHARLES A. SHULL, of Chicago, introduced the speakers and led the discussion after the papers had been presented. Prepared papers were read by Dr. OTIS F. CURTIS, of Cornell, Dr. F. G. GUSTAFSON, of Michigan, and Dr. B. S. MEYER, of Ohio, all teachers of large classes in large universities, and Dr. CHARLES J. LYON, of Dartmouth, who has small classes in an Arts College. The good attendance indicated a real interest in the seminar because it was scheduled as the last session of the society of plant physiologists, with everybody tired.

Each of the four speakers introduced by the chairman used about twenty minutes to explain the ideals and procedure by which plant physiology is taught at the institutions represented. Of the three objectives we outlined at Kingston, the information angle was assumed and the scientific attitude was emphasized only as the speakers showed how it is encouraged by oral discussions that promote accurate thought and logical reasoning. Each teacher described the mechanics of his course, the nature of the students and the level at which the topics are treated in the general, elementary course. There was marked agreement in most respects. The greatest differences appeared between the college and the university, and in the emphasis which individual teachers place upon such points as write-up of experiments, the preparation of solutions, the use of text assignments, and the critical interpretation of experimental results. No one claimed that his classes performed accurate, quantitative experiments, dipped far into chemistry, or covered the field of plant physiology. Semester courses were the rule, with the understanding that future plant physiologists need further training in advanced courses.

Some of the practices mentioned by the speakers are indicative of current trends. At Michigan, Dr. F. G. GUSTAFSON has a class of 25 to 40 with a mixture of forestry, horticulture, and Ph.D. interests. The course has a schedule of 8 hours per week that allows him to start with growth and have the students carry their own plants throughout the semester. The laboratory experiments are written up in full, but the results are also used for unannounced group discussions during laboratory hours. At Ohio State, Dr. MEYER has a class that runs very large—100 or more—for the two-quarter course (22 weeks). The students have a great variety of interests, with

<sup>1</sup> Abbreviated from a report to the New England Section at Orono, Maine, May 13, 1938.

agriculture predominant. The course is of necessity carefully planned about a series of selected assignments, lectures, and group discussions at crucial moments, and the printed laboratory manual with which you are familiar.

The story of a class of 6 or 8 Dartmouth men, handled by a modified tutorial system, contrasted with the accounts for Ohio and Michigan before, and with that for Cornell to follow. Emphasis was placed on the shortage of enthusiasm for a complex subject like ours, the total lack of a supporting graduate or professional training school, and the usual difficulties in providing the expensive quarters and equipment for a relatively unimportant elective course in a biology department. The general procedure of lecture *following* an organized but informal laboratory study of a topic was distinctive because it was so unlike the methods described for university courses. Oral examinations outside of class hours were mentioned as another instance of special devices that are possible in small classes.

At Cornell the semester course is taken by 50 to 70 students in laboratory sections that are limited to 24. In spite of the large enrollment and the need for a highly organized procedure, Dr. CURTIS emphasizes the scientific method and the critical interpretation of experimental evidence. Class experiments are limited to simple combinations of plants and apparatus, designed or selected to make the student learn a maximum number of principles with a minimum number of laboratory hours. Probably all teachers would claim the same objective but from all available evidence it appears that Dr. CURTIS has given the point rather more attention than other teachers whose methods have been described in detail.

The one method emphasized by the three university teachers and used in different form by the other speaker at Indianapolis, is the problem question as the basis of class discussion. Dr. CURTIS, Dr. GUSTAFSON and Dr. MEYER pass out mimeographed sheets of questions and require the students to work out the answers. Many of the questions are only the usual review questions, such as:

What conditions affect stomatal movement? or,

What are the differences between fermentation and respiration?

The other type of question is considered more nearly ideal for teaching students useful plant physiology. Here are a few examples:

What occupies the space between the protoplasm and the cell wall when a cell is plasmolyzed by a sucrose solution?

Cells on the convex side of a bending shoot were found to have a lower osmotic concentration, a lower turgor pressure and a higher suction tension than corresponding cells on the concave side of the same stem. What explanation can you offer?

In using a burette respirometer containing germinating seeds, what is measured by the change in level of the hydroxide solution?

If you wished to hasten the removal of carbohydrates from a given leaf, how could you do it?

Following a brief summary by Dr. SHULL, the open discussion of the evening was free and lively but it included a variety of unrelated matters and most of the questions were left unsettled. They included: definition of osmotic pressure; level *vs.* content in elementary courses; value of having the student grow experimental plants; use of fungi for experiments; and the possibility of students doing work in the nature of original research. Only the necessity of connecting with late trains stopped the session at this point; but for some reason the point of problem questions was not brought up again. To me this appeared to be the *most important single method described during the symposium*. Essentially it is the assignment of a topic for the student to explain.

As I have thought about it since, I am so impressed by its possibilities, its theoretical values, its adaptability to different institutions and also by its limitations, that I have suggested it to the officers of this section as a topic for the next symposium we conduct in the field of teaching methods.

DEPARTMENT OF BIOLOGY  
DARTMOUTH COLLEGE



## NOTES

**Ottawa Meeting.**—The summer meeting of the American Society of Plant Physiologists was held at Ottawa, Canada, June 28 to 30, 1938. Joint sessions were held with the American Phytopathological Society, and the Genetic Society of America. In the first of these joint meetings microelements and deficiency diseases were emphasized in studies of boron deficiency, spectrographic methods of detecting microelements, histological and cytological changes accompanying microelement deficiencies, and studies of thallium in relation to frenching. The symposium on microelements also considered the relation of trace elements, including iodine, copper, cobalt, manganese, and toxic levels of selenium and fluorine, to animal physiology.

In the second symposium, which occupied all of June 29, drought relations were the subject of discussion. The morphology and physiology of wheat plants grown with reduced water supply were given detailed consideration, and the similarities and differences between drought and frost resistance were discussed. Transpiration and lag in water absorption to meet the transpiration demand were emphasized, the lag being ascribed to resistance in the root tissues rather than in the stems. The water economy of trees in relation to drought also received attention, and the effect of cultivation on soil moisture relations. During the afternoon, methods of breeding drought resistant plants were reviewed, with special reference to wheat breeding and crosses between *Triticum* and *Agropyron*, and studies of the roots of wheat and other field crops. Apparatus for studying drought resistance under controlled conditions was described.

The Thursday morning session was given over to short reports of investigations of general interest. Assay methods for growth promoting substances, progress in the practical utilization of hormones, limiting concentrations of  $\text{SO}_2$  for plant development, and the effect of  $\text{SO}_2$  on the growth of conifers, apparatus for light, temperature and humidity control, and studies of cell permeability in relation to fungus parasites were among the topics presented.

On Tuesday afternoon a visit to the Central Experimental Farm at Ottawa was enjoyed by all of the plant science groups. In connection with this inspection trip, all visitors were guests of the Farm at a tea later in the afternoon. Thursday afternoon afforded opportunity to visit the laboratories of the National Research Council. Visitors were conducted through these laboratories by Dr. ROBERT NEWTON, Director of the Division of Biology and Agriculture.

On Wednesday, at noon, the society luncheon was held at the Chateau Laurier. Short talks were made by president O. F. CURTIS, vice-president

elect GEORGE W. SCARTH, and past presidents BURTON E. LIVINGSTON and W. E. TOTTINGHAM. Dr. ROBERT NEWTON served as toastmaster.

This was the eighth summer meeting, and was a very successful and satisfactory one, thanks to the efficient service of the program committee headed by GEORGE W. SCARTH, of McGill University. The reports of the secretary-treasurer, Dr. F. P. CULLINAN of the United States Department of Agriculture, showed healthy and steady progress in support.

**Richmond Meeting.**—The fifteenth annual meeting will be held at Richmond, Virginia, on December 28 to 30, 1938. The Hotel Jefferson has been designated as headquarters, and the meeting rooms for the sessions are located in the same hotel, a most satisfactory arrangement. The usual practice of holding the annual dinner on the first day, December 28, will be followed and the program committee requests that members and friends secure their tickets for the dinner promptly on arrival at the time of registration. The dinner is open to all who care to attend, and the announcement of the award of the CHARLES REID BARNES life membership, the HALES award, and the retiring president's address will make the occasion memorable. A session devoted to the problems of teaching plant physiology is planned for the closing hours on Friday.

**Dedication.**—This number of PLANT PHYSIOLOGY is dedicated to Professor FRANCIS ERNEST LLOYD in honor of his seventieth birthday anniversary on October 4, 1938, as a mark of our deep appreciation of his life-long service to plant physiology, and of his early service to the American Society of Plant Physiologists as president of the society. The following appreciation, prepared by Professor LLOYD's long-time colleague at McGill University, will find an echo in the hearts of everyone who has had the privilege of knowing Professor LLOYD and his important contributions to science. Professor LLOYD was awarded the second CHARLES REID BARNES life membership in the society at the Nashville meeting in 1927 in recognition of his valuable work.

**Francis Ernest Lloyd.**—The career of emeritus Professor F. E. LLOYD, who reached the age of 70 on October 4th, displays versatility and breadth of interest as well as an occasional radical change of outlook which may be compared to conversion. Indeed it was this experience in the orthodox sense of the term that sent LLOYD as a young man to Princeton to study for the ministry; but soon another change of heart turned his enthusiasm from theology to biology. In his earliest publications he appears as a biologist of the old school; catholicity of interest is reflected in the subjects dealt with, *viz.*, boring molluscs, bits of anatomy and taxonomy, and, in a more impor-



tant paper, the embryology of the Rubiaceae. Some glimmerings of a new light are seen in this last paper which gives to the anatomical facts a physiological explanation. But LLOYD's road to Damascus only brought him to the crisis when he reached the Arizona desert. "Those were the days," the early days of the Tucson laboratory, the stimulation of its physical and mental atmosphere and the new vision which converted our old-fashioned botanist into an ultra-modern physiologist, one to whom teleology was anathema and mechanism an inspiring creed! *The Physiology of Stomata* published at this period is notable not only for its positive contributions to the knowledge of stomatal mechanism but also for the iconoclastic glee with which the teleological assumption of perfect regulation of transpiration by stomata is discredited. As was predicted by FRANCIS DARWIN at the time, this work proved highly stimulative and it is perhaps the most quoted of all LLOYD's publications.

The new recruit to physiology was soon marching in the van of the movement, applying biophysics to the physiology of the cell. In this strain is his work on the fate of tannin during the ripening of fruits—not oxidation, he found, but adsorption by another colloid; and again on the growth of pollen tubes in acid and alkali which he showed to run parallel to the swelling of gelatin. The biophysical approach became the evangel of his teaching and the elementary course in General Physiology which he developed in later days at McGill was even then a pioneering effort.

Though a fundamentalist at heart LLOYD frequently entered the field of applied research. His monograph on Guayule and its methods of culture which formed the basis of later practical developments, and his correlation of boll-shedding of cotton with a sinking of the water table (under Alabama conditions, at least) are examples of direct attack on practical problems.

But his best work dealt with topics of which the interest is primarily academic, such as the conjugation of *Spirogyra*, especially the rôle of contractile vacuoles in the process, and the varied functions of carnivorous plants, especially the trap mechanism of *Utricularia*. It is subjects such as these that particularly suit LLOYD's talent—subjects possessing a certain dramatic interest and requiring for their study infinite patience, technical dexterity, and keen observation. Though early in the biophysical field, his training was hardly such as to enable him to push that type of investigation to the utmost—much to his regret. He is wont to say that his only training was listening to the dictum of his first and much beloved biology teacher, Professor GEORGE ("Pat") MACLOSKE of Princeton University: "Nature is the highest authority." This explains, perhaps, the seemingly erratic course which his researches have pursued. Ever questioning nature he followed whatever lead she gave him. He dipped wherever he saw a fish.

One ruling passion, however, has persisted through all the turns and twists of LLOYD's career, an insatiable curiosity to discover how a piece of

mechanism works. The same desire "to see the wheels go round" that apprenticed LLOYD as a youngster to the watchmaking trade still burns strong in old age and infirmity as he probes the beautiful mechanism of the bladderwort trap. Nature is having her revenge! The youth who scoffed at the adaptations of stomata has lived to become the mature interpreter of one of the most perfect and puzzling adaptations in the plant kingdom. Perhaps a final conversion is due!

Not the least of LLOYD's contributions to physiological science is the arousing of public interest through his lectures. Possessed of a strong dramatic sense, he could tell the story of his researches with effect; and artistically gifted, he illustrated his narrative with beautiful hand-coloured slides and motion pictures which are known the world around.

LLOYD is a cosmopolitan. He has carried on research and given his lectures in all parts of North America, many European countries and all the continents. Now he enjoys a busy rest at Carmel, California, working daily in the old coastal laboratory of the Carnegie Institution of Washington amid scenes and associations that recall the days when physiology claimed him for her own.—GEORGE W. SCARTH.

**Errata.**—A small list of errors in volume 13 of PLANT PHYSIOLOGY is printed at the close of the table of contents. It is advisable to record the corrections appropriately at the places where the errors occur, so as to avoid misinterpretations. The editor solicits the aid of all authors in detecting and correcting errors which occur in their papers. We do not have time to proof read the printed volumes, and appreciate the valuable aid extended by those who reported mistakes.

**Minor Elements.**—Because of the rapid exhaustion of previous editions of the *Bibliography of References to the Literature on the Minor Elements and Their Relation to Plant Nutrition*, we are making a pre-publication announcement of the early appearance of a third edition of this invaluable reference bibliography. It is to be issued in January, 1939, by the Chilean Nitrate Educational Bureau, Inc., 120 Broadway, New York. As long as the supply lasts copies will be available at \$1.00 per copy. This edition will carry about 4700 abstracts and references, and it is expected to be the last complete edition the Bureau will undertake to publish. Members are urged to place orders promptly with Mr. HERBERT C. BREWER, Director, at the address given. Every member of the society should possess a personal copy of this annotated bibliography.

**Outlines of Biochemistry.**—A welcome addition to the literature of biochemistry is the second edition of Dr. R. A. GORTNER's *Outlines of Biochem-*

*istry*, published by John Wiley and Sons. The work has been carefully rewritten, and chapters added dealing with oxidation-reduction, the flavins, and the hormones. It is brought up to date in such progressive fields as the physiological chemistry of the vitamins, sterols, and other hormones. Plant physiologists would profit by making this splendid volume a daily companion. It is listed at \$6.00 per copy.

**Chromosome Structure.**—A new volume in the Protoplasma-Monographien series, no. 14, is Dr. LOTHAR GEITLER's (University of Vienna) *Chromosomenbau*. It is a technical discussion of the structure of chromosomes, presented in six chapters. It deals not only with the external features of chromosome morphology, but also with the microscopic and sub-microscopic structures. It is not intended for beginners, but for those already well versed in the knowledge of chromosomes, and their behavior in all kinds of cells. Cytologists and geneticists will find it valuable in bringing together and summarizing a large field. About 700 titles are included in the literature list. The work is well illustrated (69 figures) and is appropriately indexed. The quoted price, bound in red cloth uniform with other volumes in the series, is RM 15. Orders may be sent to Gebrüder Borntraeger, Berlin.

**Leaf Xanthophylls.**—An excellent monographic study of the leaf xanthophylls has been published by the Carnegie Institution of Washington as Publication no. 490. The author is Dr. HAROLD H. STRAIN, of the Institution.

The introduction gives an account of the methods of separation of the numerous xanthophylls from one another, the molecular structure of the carotenes and xanthophylls, nomenclature, physiological functions, state of the pigments in leaves, and their oxidation in killed leaves.

The following section deals with the principal properties of 13 carotenes and 30 xanthophylls. Three xanthophyll esters and 7 carotenoid acids are described, and a lone unclassified member, rhodovibrin.

The experimental techniques require great skill, and involve extraction, isolation and separation in pure form, and determination of the main properties, such as absorption spectra, melting points, optical properties, empirical formulae, color reactions, microscopic examination, and quantitative estimation of leaf xanthophyll.

The results of these examinations occupy the major portion of the monograph. Chromatographic adsorption has proved an invaluable aid in the separations. A frontispiece in color illustrates the method used in this work. The xanthophylls turn out to be mainly mono-, di-, tri-, and tetra-oxo derivatives of carotenes, with lutein the main constituent in a mixture

of a dozen or more xanthophylls in any given sample. Crystallization from petroleum ether prevents the main losses incident to the WILLSTÄTTER and STOLL procedures. The entire monograph reflects the skill and patience with which the problems have been approached. The fact that only partial success has been attained, especially in the quantitative aspects of the work, emphasizes the difficulties encountered all along the line. The contribution is a very worthy one, and plant physiologists will be grateful for the insight which the publication gives into one of the difficult phases of plant biochemistry. A bibliography of 190 titles, and author and subject indexes make it useful in connection with past work. It deserves a place in the libraries of all who are interested in plant pigment chemistry.

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